Antioxidant activities of underexplored Chinese medicinal plant parts and their effect against high glucose-induced modulation of fibronectin expression

B Sridharan¹, Y X Zhong¹, Y K Rao¹, Y M Tzeng¹, M J Lee¹, ²

¹ Department of Applied Chemistry, Chaoyang University of Technology, 168 Jifeng East Road, Taichung, 413310 Taiwan, R.O.C.

² E-mail: mjlee@cyut.edu.tw

Abstract. Diabetes has been a long-standing disorder and its management has been challenging various medical and research experts for several decades because of its complex causative factors and pathophysiological processes leading to complications. Medicinal plants have been explored in several countries and traditional Chinese medicine is one of the well-recognized alternative treatment methods. In this study, we have chosen some of the underexplored plant parts of Chinese medicinal herbs and analyzed their antioxidant activity and ability to modulate the expression of fibronectin during high glucose conditions. Extraction of the plant materials with different solvent led to 17 extracts and among which, 3 extracts (2, 12 & 17) were observed to render more than 50 µg/ml vitamin C equivalents of DPPH free radical scavenging ability and 2 of them (2 & 17) showed more than 25 µg/ml of vitamin C equivalents of ferric ion reducing power. Based on the antioxidant activity and comparison of their total phenolic content, we used extracts 2 & 17 to check their effect on fibronectin expression in MES-13 cells under high sugar conditions. We observed that both extracts showed a significant reduction of fibronectin expression compared to untreated cells with high glucose levels. The expression was much lesser than the normal untreated, normal sugar supplemented cells and this was not observed in vitamin C supplemented cells. In conclusion, crude extracts containing a group of phenolic compounds have shown significant effects against fibronectin expression leading to reduced ECM deposition and tissue fibrosis. Further exploration might provide insights into the exact mechanism and checkpoints of the extract that can successfully reduce diabetes-induced renal complications.

1. Introduction
Diabetes Mellitus is one of the complicated disorders, that is characterized by chronic hyperglycemia [1], and the pathological conditions induced by diabetes include diabetic retinopathy, nephropathy, neuropathy, and diabetes-induced cardiovascular complications [2, 3]. All diabetes-mediated complications are due to high glucose content and excessive glycosylation of functional proteins, leading to cellular conditions like mitochondrial dysfunction, reactive oxygen species (ROS) generation, apoptosis, and extracellular matrix (ECM) deposition leading to fibrosis [4-6]. Diabetes-induced renal complications are very common next to retinopathy and pathologically it is characterized by glomerulosclerosis, tubular necrosis, podocyte loss, and tissue fibrosis [7]. It is a herculean task to target all the predisposing factors and complications of diabetes with a single synthetic/natural compound [8]. This could be the reason that most of our ancestors relied on extracts obtained from medicinal herbs that
contain a group of bioactive compounds that can act together to render optimal activity against disease progressing mechanisms [9, 10].

Figure 1. Different plant materials used in this study. Grape seeds (A; https://www.feedipedia.org/node/692), Grape skins (B; https://www.shutterstock.com/), Antrodia Campharata (C; https://www.taiwantrade.com/), Cinnamomum osmophloeum Kaneh (D; https://lovelytaian.org/), Cordyceps Sinensis (E; https://hifasdaterra.co.uk/), Phyllanthus urinaria L. (F; https://plants.ces.ncsu.edu/).

Traditional Chinese medicine is part of alternative medicine-based therapy and is been well recognized among other traditional medicine practices in various countries. Several plant species and their plant parts were been explored and reported for their ability to inhibit several diseases and their complications [11, 12]. In our study, we have selected some of the under-explored plant parts (Figure 1) such as skin and seeds of the grape, Antrodia Campharata (Fruit bodies), Cordyceps Sinensis (Fruiting bodies), Cinnamomum osmophloeum Kaneh (bark), and Phyllanthus urinaria (Leaves). We extracted these plant materials with several organic solvents ranging with different polarities to obtain their polyphenolic contents. The obtained crude extracts were tested for their ability to scavenge free radical (DPPH assay) and reduced ferric ion (FRAP assay). The extracts with the best activities were chosen and their ability to inhibit fibronectin expression during high sugar condition was analysed in MES-13 renal mesangial cells, to mimic the diabetic nephropathy condition in vitro.

2. Materials and methods

2.1. Materials
All the chemicals and reagents used in this study were as purchased from Merck Taiwan Co., Ltd. MES-13 cell lines were purchased from Bioresource Collection and Research Centre, Food Industry Research and Development Institute, Hsinchu, Taiwan.

2.2. Source of crude plant extracts
There are a total of 17 extracts obtained from 6 different plant parts: grape skins, grape seeds, Antrodia Campharata (AC) fruiting bodies, Taiwan soil cinnamon bark (Cinnamomum osmophloeum Kaneh; CO origin: China), Cordyceps Sinensis (CS) fruiting bodies, Phyllanthus urinaria L. (PU). The plant materials were selected out of commercial interests. The grape skins and seeds were from the left over
of wine process, and were obtained from the local winery. PU is of great interest because it has high antioxidative potential, and was collected in the campus of CYUT; AC, CO and CS were popular health product in Taiwan. CO was provided by farm located in Hua-lien County, Taiwan. AC was grown on a log in CYUT for a local biotech company. CS was purchased from the local Chinese herb pharmacy.

2.3. Free radical scavenging ability test (DPPH scavenging test)
The ability of extracts to scavenge DPPH free radicals was measured by the method described by Shimada et al., 1992 [13]. Briefly, 0.6 mL of crude plant extract solution (100 μg/ml), and 1.2 mL of freshly prepared 0.4 mM DPPH solution using methanol were mixed well and incubated for 30 minutes. The absorbance was measured at 517nm. The lower the absorbance value indicates stronger the DPPH scavenging ability. The percentage of activity was calculated using the formula given below and compared with the standard curve of L-ascorbic acid to calculate the free radical scavenging ability of the sample relative to Ascorbic acid.

2.4. Determination of total antioxidant capacity (FRAP assay)
Ferric ion reducing power was measured according to the method of Reka Szollosi [14]. Briefly, 60μl of crude plant extract solution (100 μg/ml) was added with freshly prepared 1.8 ml FRAP solution (300 mM Acetate buffer (pH = 3.6); 10 mM TPTZ in 40 mM HCl; 20 mM FeCl3.6H2O = 10:1:1). The contents were mixed evenly and the absorbance was measured after 5 mins at 593 nm. The data was compared with the standard curve of L-ascorbic acid and the total antioxidant capacity of the sample relative to L-ascorbic acid was obtained.

2.5. Determination of total phenolic compounds (Folin-Ciocalteu’s test)
Estimation of the total phenolic content in the extracts was performed according to the Folin-Ciocalteu method, described by Kumar et al [15]. Briefly, 80μl of crude plant extract solution (100 μg/ml) was added with 720 μl of deionized water and 80μl of Folin-Ciocalteu's reagent and mixed evenly. Then 800 μl of 7% Na2CO3 solution was added and incubated for 5 minutes. The volume of the content was adjusted using 2 ml of deionized water and absorbance was measured at 750 nm after 90 minutes at 23°C. The results were compared with the standard curve of L-ascorbic acid and total phenolic compounds in the sample were expressed relative to the ascorbic acid equivalents.

2.6. Cell culture
The MES 13 cell line was purchased from the Institute of Food Industry Development (Hsinchu, Taiwan) and cultured at 37°C under 5% CO2. The composition is of the media includes Ham's F12 = 3: 1, 1 g/L glucose, 5% FBS, 14 mM HEPES, 1% three-in-one antibiotics, and the cells were grown about 8 minutes full. Then the medium was changed with serum-free medium (Serum-Free medium; SFM) and cultured for 2 days to make the cells enter the resting phase. Finally, glucose concentration was changed to 1 g/L or 5 g/L of SFM and cells were cultured with different concentration of vitamin C, grape seed extract and Phyllanthus urinaria extract for 2 days. After 2 days, the respective culture medium was collected for protein analysis using western blot.

2.7. Western blot
The culture medium was dried in a freeze dryer, and then re-dissolved in deionized water. The protein concentration was measured using the Bio-Rad Protein Assay Kit. The concentrated culture medium and an equal volume of sample buffer containing 50mM Tris-HCl, 2% SDS, 0.1%, Bromophenol blue, 20% Glycerol, and 100 mM 2-Mercaptoethanol were mixed, and the contents were heated at 100°C for 5 minutes before separating the proteins in the SDS-PAGE gel. The gel contained 5% Stacking gel and 10% Running gel for protein separation, and pre-stained SDS-PAGE standard (broad range, Bio-Rad) was used as the molecular weight marker. After the electrophoresis, proteins were transferred to the PVDF membrane and anti-Fibronectin antibody was added and incubated overnight. On the following day, the membrane was incubated with secondary antibody for 1 hour. Finally, the Amerşhan ECL kit
was used for the reaction, and chemiluminescence was captured on X-ray film directly. The results obtained were analyzed using Image J software and expression of the protein was reported as a ratio of intensity of the protein band to the total protein concentrated added to the gel.

3. Results

3.1. Antioxidant activities
The plant extracts prepared using different solvents were tested for their ability to scavenge the DPPH free radicals and Ferric reducing power. They were displayed in Table 1. To support the amount of antioxidant activities observed in the extract, we have checked the phenolic content in each extract.

Table 1. Antioxidant activities of crude extracts obtained from all the plant materials.

| Extract No | Crude extract (100 µg/ml) | Antioxidant activities | Sample concentration (100 µg/ml) |
|------------|---------------------------|------------------------|----------------------------------|
| 1          | Grape seed Chloroform     | DPPH radical scavenging activity | 25.5          |
| 2          | n-Butanol                 |                        | 20.0          |
| 3          | Water                     |                        | 0.0           |
| 4          | n-Hexane                  |                        | 5.1           |
| 5          | Grape skin n-Butanol      |                        | 6.3           |
| 6          | Dichloromethane Ethyl acetate | FRAP assay | 2.5     |
| 7          | Ethyl acetate             |                        | 23.1          |
| 8          | n-Hexane                  |                        | 5.7           |
| 9          | Antrodia Campharata       |                        | 3.4           |
| 10         | Methanol                  |                        | 5.6           |
| 11         | Cinnamomum osmophloeum Kaneh. Ethyl acetate | Total phenolic content | 15.2  | 8.9 | 0.0 |
| 12         | Methanol                  |                        | 50.6          |
| 13         | n-Hexane                  |                        | 0.0           |
| 14         | Cordyceps Sinensis Chloroform | Total phenolic content | 5.7  | 0.9 | 0.0 |
| 15         | Phyllanthus urinaria L. n-Hexane | Total phenolic content | 3.7  | 1.4 | 0.0 |
| 16         | Chloroform                |                        | 3.9           |
| 17         | Methanol                  |                        | 85.8          |

a Relative antioxidant activity of the extract with respect to vitamin C (µg/ml)
b Relative phenolic content in the extract with respect to vitamin C (µg/ml)

DPPH radical scavenging assay: Crude extracts of different plant materials showed activities around 2 - 20 µg/ml relative to vitamin C which was found to be very ordinary and it was obvious from the Folin-Ciocalteu’s assay for total phenolic contents, where none of the extracts except 2, 12 & 17 showed the presence of a significant number of phenolic compounds. The extracts 2, 12 & 17 showed significantly high amount of DPPH radical scavenging activity relative to µg/ml of vitamin C (96.3, 50.6 & 85.8 respectively) with high amount of phenolic content (7.6, 0.4 & 0.4 µg/ml ascorbic acid equivalents). This shows among 17 crude extracts, these three extracts show the ability to donate a pair of electrons and scavenge the free radicals.

FRAP assay: The ability of a compound or group of compounds to protect cells from free radical-induced damage was recognized by its ability to reduce ferric to ferrous form of iron. The plant extract explored in this study showed minimal ferric reducing power relative to vitamin C (µg/ml) except
extracts 2 (53.6), 12 (16.1) & 17 (25.8) as observed in DPPH based assay. The Ability of other extract was observed less than 15% of ferric reducing power compared to vitamin C (Table 1).

3.2. Total phenolic content
The biological activity of plant extract mainly depends on the amount of phenolic content it contains and the extracts that showed a relatively high percentage of antioxidant activities observed to possess a higher number of phenolic compounds compared to other extracts. Hence, we used extracts 2 & 17 to check their effect on the expression of fibronectin, which possesses a crucial role in the wound healing process.

3.3. Comparison of antioxidant activities and total phenolic content
When we compared the antioxidant activities of all the extracts, 2, 12 & 17 extracts showed the best activity against DPPH radical & ferric ion reducing power with its high phenolic content (Figure 2). Other than these extracts, 3, 7 & 11 showed more than 10 µg/ml of vitamin C equivalents of DPPH radical scavenging activity, while extracts, 1, 5 & 7 showed more than 10 µg/ml of vitamin C equivalents of ferric ion reducing power. However, only 2, 12 & 17 extracts showed the presence of significant level of phenolic compounds relative to ascorbic acid equivalents, while other extracts failed to show detectable phenolic contents.

3.4. Western blotting
When we supplemented the cells with plant extracts with better antioxidant activity (such as Phyllanthus urinaria or grapeseed), we can find that the amount of fibronectin secreted by the cells was reduced, much lower than the normal cells untreated with extract of high glucose content. It shows that both the extracts protected cells from damage induced in a high-sugar environment (Figure 3). In comparison with vitamin C with the same antioxidant capacity, the expression of fibronectin was not reduced. This showed extract contained different molecules that acted on the signaling pathways responsible for fibronectin regulation which was not achieved by vitamin C alone. It can be seen from Figure 3 that when 8.6 µg/ml of grape seed (n-butanol) extract was added, fibronectin was significantly downregulated, but this phenomenon needs to be further explored for its exact mechanism of action.
Figure 3. Western blotting analyses of Fibronectin. Protein bands observed after treatment against samples (A); Graph showing the difference in intensity of the bands observed in ImageJ software (B). PU indicates *Phyllanthus urinaria* and GS indicates grape seed. Concentration of the samples: Vitamin C – S – 0.086 µg/ml; 10S – 0.86 µg/ml; 100S – 8.6 µg/ml. PU – S – 0.1 µg/ml; 10S – 1 µg/ml and 100S – 10 µg/ml. GS – S – 0.089 µg/ml; 10S – 0.89 µg/ml; 100S – 8.9 µg/ml. The results were statistically analyzed by Student t-test (n=3) and comparisons were made as *High glucose treatment Vs Normal glucose treatment (* - p<0.05); # High glucose + Sample (100S) treatment Vs High glucose treatment (# - p<0.05).

4. Discussion

Complementary and alternative medicine was always sought by more than 3/4th of the general population and the World Health Organization (WHO) reported that traditional medicines were sought 2-3 times more than synthetic drugs [16-18]. Chinese medicinal plants have gained significant interest due to their diverse biological properties owing to their polyphenolic content present in different parts of those plants [11]. These plants and plant parts were used to treat many metabolic disorders like diabetes, neurodegenerative disorders, cancer, etc., and also inhibit infectious diseases causing organisms like HIV, HBV, including bacterial and fungal organisms [19]. Among various metabolic diseases, diabetes has been posing a significant burden on the hospital and research community, and several synthetic and natural compounds are being tested for management of diabetes but the complications arise in the system due to diabetic mechanism is very intricate and a single compound cannot be successful in targeting a variety of mechanisms involving diabetes [8, 20]. Hence, we tested medicinal plants’ extracts containing a group of phenolic compounds in our study. Several well-known plant species are popular for their ability to reduce the progression of various metabolic diseases and we have used some of the under-explored plant materials like epicarp and seeds of the grape, fruiting bodies of *Antrodia Campharata, Cordyceps Sinensis*, Taiwan soil Cinnamon bark and leaves with fruits of *Phyllanthus urinaria*. The plant materials chosen in our study were extracted with a variety of organic solvents (from polar to non-
polar) based on literature and 17 crude extracts were chosen and explored in the study according to the yield. 

Our primary goal is to exhibit the antioxidant property of the plant extracts to show that ROS and its pertaining mechanisms can be targeted by these extracts. The results confirmed the ability of butanol extract of grape seed (2), Methanol extracts of Cinnamomum osmophloeum. Kaneh (12) and Phyllanthus urinaria. L (17) was significantly higher compared to other extracts. Among these 3 extracts, 2 & 17 showed more than 80 µg/ml vitamin C equivalents of DPPH radical scavenging ability and more than 20 µg/ml vitamin C equivalents of Ferric reducing power. The phenolic contents of these 2 extracts were also at a higher level compared to other extracts. However, the FRAP assay showed extract 1 also showed 20 µg/ml vitamin C equivalents of activity, but the phenolic content and DPPH scavenging ability were not at the satisfactory level. Also, extract 12, though had more than 50 µg/ml vitamin C equivalents of DPPH scavenging ability, its FRAP activity was less than 20 µg/ml vitamin C equivalents and it was not used to check its ability to inhibit fibronectin expression though it showed detectable levels of phenolic compounds in it. Several compounds were already isolated and reported from the plant materials used in our study. Grape seed and skin were explored and reported for presence of gallic acid, epicatechin, ferulaic acid, cyanidin-3-glucoside, and etc [21]. Terpenoids such as Antrocin and Antcin derivatives, bioactive sterols, derivatives of antrocamphins, antroquinonol derivatives are some of the structurally elucidated compounds from Antrodia Campharata [22]. Cordyceps Sinensis contains cordycepin, cordycepic acid, corysinocan and many sterol derivatives, while cinnamon bark is mainly composed of cinnamaldehyde and eugenol [23, 24]. Phyllanthus urinaria L consists of phyllanthin, urinatetralin, hypophyllanthin, phyltetralin, and etc [25].

Figure 4. Effect of high glucose condition on fibronectin and extracellular matrix overexpression [7, 28-30]

In our study, we considered antioxidant activity as the primary property of the extract to carry out further exploration, because, a group of phenolic compounds synergistically act against several disease progressing mechanism and most of the diseases such as diabetes, cancer, etc., rapidly progresses due to ROS mediated mechanisms [26]. During diabetes, renal complications are very common and the kidney is one of the highly affected organs (20-40%) next to the retina in diabetic patients [27].
Pathological events like glomerulosclerosis (mesangial expansion, basement membrane thickening and nodular glomerulosclerosis), tubular changes (tubular hypertrophy and necrosis), and fibrosis (interstitial fibrosis and tubular atrophy) are major characteristics of diabetic nephropathy [7, 28]. Among various biomolecules responsible for diabetes-induced renal damage, the formation of advanced glycation end products is very prominent and it increases the TGF-β mediated fibronectin expression and renal fibrosis [29]. On the other hand, high glucose level also leads to mitochondrial dysfunction and ROS generation leading to NF-κB induced caspase 3 upregulation and apoptosis [30]. In our study, we checked the expression of fibronectin at high glucose environment supplemented with the extracts and results clearly showed that the expression of fibronectin was reduced and only possible inference out of this observation was that, the group of compounds inhibited the ROS and reduced AGE & PKC induced ECM deposition through downregulation of fibronectin (Figure 4). This observation opens up a huge venture in the area of diabetic nephropathy and further exploration can help in the identification of the compounds and its exact mechanism by which the renal pathological events were inhibited.

5. Conclusion
In summary, the antioxidant activities of some of the important Chinese medicinal herbs were explored and we found that among various crude extract, butanol extract of grape seed and methanolic extract of *Phyllanthus urinaria* showed significant DPPH free radical scavenging ability and ferric reducing power. Further, we presumed that ROS-mediated mechanism in diabetes-induced renal complications can be reduced with the help of these 2 extracts. We mimicked the diabetic condition, *in vitro* by supplementing a high amount of glucose to renal mesangial cells (MES-13) and added crude extracts. We observed that expression of fibronectin at high glucose environment was significantly reduced by the extracts and the activity was higher compared to the ability of vitamin C. Hence, we conclude that Chinese medicinal plants and their parts possess high amounts of polyphenolic compounds and they work synergistically to inhibit vital pathological processes in diseases such as diabetes. Hence, meticulous exploration of such extracts obtained from underexplored Chinese medicinal plant parts will provide a better path towards the management of multifactorial diseases.

References
[1] Paul S, Ali A and Katare R 2020 *J. Diabetes Complications* 34 107613
[2] Volpe C M O, Villar-Delfino P H, Dos Anjos P M F and Nogueira-Machado J A 2018 *Cell Death Dis.* 9 1-9
[3] Preguiça I, Alves A, Nunes S, Gomes P, Fernandes R, Viana S D and Reis F 2020 *Nutrients* 12 250
[4] Chatham J C, Young M E and Zhang J 2021 *Curr. Opin. Pharmacol.* 57 1-12
[5] Giri B, Dey S, Das T, Sarkar M, Banerjee J and Dash S K 2018 *Biomed. Pharmacother.* 107 306-28
[6] Ravichandran G, Lakshmanan D K, Murugesan S, Elangovan A, Rajasekaran N S and Thilagar S 2021 *Food Res. Int.* 140 110081
[7] Djudjaj S and Boor P 2019 *Mol. Aspects Med.* 65 16-36
[8] DeFronzo R A, Ferrannini E, Groop L, Henry R R, Herman W H, Holst J J, Hu F B, Kahn C R, Raz I and Shulman G I 2015 *Nat. Rev. Dis. Primers* 1 1-22
[9] Gothai S, Ganesan P, Park S-Y, Fukurazi S, Choi D-K and Arulselvan P 2016 *Nutrients* 8 461
[10] Chang C L, Lin Y, Bartolome A P, Chen Y C, Chiu S C and Yang W C 2013 *Evid. Based Complement. Alternat. Med.* 2013 378657
[11] Fung F Y and Linn Y C 2015 *Evid. Based Complement. Alternat. Med.* 2015 425037
[12] Lu A-P, Jia H-W, Xiao C and Lu Q-P 2004 *World J. Gastroenterol.* 10 1854
[13] Shimada K, Fujikawa K, Yahara K and Nakamura T 1992 *J. Agric. Food Chem.* 40 945-8
[14] Szöllösi R and Varga I S I 2002 *Acta Biol. Szeged.* 46 125-7
[15] Kumar K and Goh K 2003 *Comm. Soil Sci. Plant Anal.* 34 2441-60
[16] Shukla Y and Pal S 2004 *Asian Pac. J. Cancer Prev.* 5 3-14
[17] Mehta P, Shah R, Lohidasan S and Mahadik K 2015 J. Tradit. Complement. Med. 5 207-27
[18] Pawar A, Rajalakshmi S, Mehta P, Shaikh K and Bothiraja C 2016 RSC Adv. 6 69282-300
[19] Yang L, Yang C, Li C, Zhao Q, Liu L, Fang X and Chen X-Y 2016 Sci. Bull. 61 3-17
[20] Song Y, Yang J, Jing W, Wang Q, Liu Y, Cheng X, Ye F, Tian J, Wei F and Ma S 2020 Chin. Med. 15 1-15
[21] Tang GY, Zhao CN, Liu Q, Feng XL, Xu XY, Cao SY, Meng X, Li S, Gan RY and Li HB 2018 Molecules 23 2598
[22] Geethangili M and Tzeng YM 2011 Evid Based Complement Alternat Med. 2011 212641
[23] Liu Y, Wang J, Wang W, Zhang H, Zhang X and Han C 2015 Evid Based Complement Alternat Med 2015 575063
[24] Rao PV and Gan SH 2014 Evid Based Complement Alternat Med 2014 642942
[25] Geethangili M and Ding ST 2018 Front. Pharmacol. 9 1109
[26] Leena M M, Silvia M G, Vinitha K, Moses J and Anandharamakrishnan C 2020 Food Funct. 11 9317-37
[27] Wu Y, Zhang C, Guo R, Wu D, Shi J, Li L, Chu Y, Yuan X and Gao J 2021 Stem Cells Int. 2021 6620811
[28] Lim AKH 2014 Int J Nephrol Renovasc Dis 7 361-81
[29] Rojas A, Añazco C, González I and Araya P 2018 Carcinogenesis 39 515-21
[30] Lee M J, Rao Y K, Chen K, Lee Y C, Chung Y S and Tzeng Y M 2010 J. Ethnopharmacol. 132 497-505