Caffeic Acid Attenuates Diabetic Kidney Disease via Modulation of Autophagy in a High-Fat Diet/Streptozotocin-Induced Diabetic Rat

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The aim of this study is to evaluate the anti-diabetic nephropathy effect of Caffeic acid and to prove our hypothesis for its mechanism of action that it may occur by reactivation of autophagy pathway via suppression of autophagy regulatory miRNAs. In vivo, high-fat diet and streptozotocin-induced (HFD-STZ) diabetic rats were treated with Caffeic acid once per day for 12 weeks before and after development of diabetic nephropathy. Blood and urine biochemical parameters, autophagy transcripts and their epigenetic regulators together with renal tissue morphology were investigated. In diabetic rats, Caffeic acid intake, caused improvement in albumin excretion, blood glucose, reduced renal mesangial matrix extension with increased vacuolation and reappearance of autophagosomes. Meanwhile, it resulted in autophagy genes up-regulation [RB 1-inducible coiled coil protein (RB1CC1), Microtubule-associated proteins 1A/1B light chain 3 (MAP1LC3B), Autophagy related gene (ATG-12)], with simultaneous reduction in their epigenetic regulators; miRNA-133b, −342 and 30a, respectively. These above mentioned effects were more significant in the diabetic nephropathy Caffeic treated rats than in the prophylactic group. Based on our results we postulated that caffeic acid modulates autophagy pathway through inhibition of autophagy regulatory miRNAs, that could explain its curative properties against diabetic kidney disease.

The prevalence of diabetic kidney disease (DKD) has been increasing world wide. Therapeutic strategies, including antidiabetic drugs and inhibitors of the renin–angiotensin, can postponed DKD. Accordingly, we need to look for a possible remedial target to treat or avoid DKD1. Recent studies highlighted the role of genetic and epigenetic mechanisms in the regulation of autophagy process as well as the pathogenesis of DKD2. Reduction of autophagy results in oxidative stress, podocyte injury, mesangial cell proliferation, glomerular endothelial dysfunction, accumulated collagen and TGF-β13. Thus, a cytoprotective multitarget modulation of autophagy is significantly required to attenuate renal damage in diabetes1. Specific miRNAs have currently been identified as significant epigenetic modifiers of autophagy linked genes. In fact, these autophagy linked genes’ mRNA includes, the target sequence for miRNAs related to diverse families5,6. The gene networks regulating autophagy pathway were determined using a system biology and unrevealed miR-130, miR-98, miR-124, miR-204, and miR-142 as presumed posttranscriptional modulators of this pathway at different levels6. Therefore, unraveling the significance of autophagy-miRNA interaction in DKD might lead the way to novel diagnostic and molecular therapeutic targets for DKD7. We have therefore focused on a strategy for

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the resumption or activation of autophagy. Proautophagic drugs are a promising class of compounds for diabetic nephropathy regression. Recent studies revealed many phytochemical constituents that can induce autophagy.

Wang and his colleagues reported that caffeic acid extract of *Artemisia dracunculus* L. enhances insulin receptor signaling and modulates gene expression in skeletal muscle in KK-Ay mice. Scientists recently reported that a semisynthetic compound derived from caffeic acid derivative induces DNA damage and apoptosis in tumor cells via induction of autophagy in cancer cells. Also, Caffeic acid derivative have been reported to have a protective role in renal damage. We sought to determine the efficacy of caffeic acid on modulating autophagy pathway via inhibiting expression of miRNA related DKD with subsequent upregulation of its target autophagy related genes utilizing HFD-STZ diabetic rats.

The rational of this study was based on: (1) Bioinformatics analysis to retrieve a set of 3 DKD-characteristic autophagy genes (RB1-inducible coiled coil protein (RB1CC1), Microtubule-associated proteins 1A/1B light chain 3 (MAP1LC3B), Autophagy related gene (ATG-12)). Then to choose miRNAs (miR-133b, miR-342 and miR-30a) relevant to DKD and acting as epigenetic regulators of the former autophagy genes based on previous microarray studies; (2) Experimental validation to characterize the efficacy of caffeic acid on modulating the expression of chosen miRNA-autophagy target gene pairs in HFD-STZ induced rat model.

**Results**

**Effect of caffeic acid on fasting blood glucose and other metabolic parameters in HFD-STZ induced diabetic rats within the first five weeks.** STZ resulted in a significant increase in blood glucose level, compared to either control or naïve group. [F = 463.8, p < 0.01], with a significant increase of blood glucose level over time [F = 319.7, p < 0.01]. Bonferroni posttest was used to compare the both groups at different time points. Blood glucose level in HFD-STZ treated group was significantly higher from the naïve group starting at week 2, and progressively increased till week 4 (P < 0.01; Fig. 1).

After 12 weeks, the levels of FBG, serum total cholesterol, LDL, total triglycerides in the DM group were significantly higher than those of the NC group. Compared with DM group, Caffeic acid treatment and prophylactic groups showed marked lowering of FBG and lipid profile indicating a clear improvement in the glucose and lipid metabolism (Table 1).

**Effect of caffeic acid on renal function of HFD-STZ induced diabetic rats.** Urinary albumin, urinary creatinine, serum creatinine, blood urea nitrogen (BUN), urea and creatinine clearance in DN group were increased compared to NC group. Dose-response effects of CA on creatinine clearance in HFD-STZ rats was performed. Increasing the dose of CA resulted in an improvement of creatinine clearance. Thus, we found that CA had a pronounced dose-dependent effect on improvement of creatinine clearance (Fig. 2) and 40–50 mg/kg of CA were the most effective doses for improving creatinine clearance in HFD-STZ rats.

The ameliorating effect of caffeic acid on renal function parameters was evident both in the treatment and prophylactic groups, (Table 1).

**Effect of caffeic acid on renal histology and autophagy induction in HFD-STZ induced diabetic rats.** Parallel to the development of DM in HFD-STZ treated rats, histopathological examination of H&E stained kidney tissues by light microscopy revealed a focal inflammatory cells infiltration as well as detection of aggregation in-between the degenerated tubules and glomeruli at the cortex in diabetic group. Moreover, experimentally induced DKD group showed severe congestion in the glomeruli associated with swelling in the tubular lining epithelium at the cortex. Furthermore, prophylactic administration of caffeic acid in HFD-STZ induced diabetic rats showed mild congestion in the glomeruli associated with swelling in the lining tubular epithelium. Lastly treatment with caffeic acid in HFD-STZ induced diabetic rats resulted in glomerular vacuolization in the lining endothelium of the tufts associated with degeneration in the tubular lining epithelium at the cortex. All in all, prophylactic and caffeic acid treated group, significantly ameliorated the renal changes with extensive vacuolation(Fig. 2A–E). To visualize autophagy induction by caffeic acid in DKD, TEM was performed. Electron micrographs of autophagosomes was demonstrated in figure (Fig. 3A–K). Rare autophagic vacuoles were detected...
|                          | Mean     | Std. Error | F     | Significance |
|--------------------------|----------|------------|-------|--------------|
| Serum creatinine concentration (mg/dl) |          |            |       |              |
| control                  | 1.00     | 0.000      |       |              |
| placebo                  | 1.00*    | 0.000      | 7.070 | 0.0001       |
| diabetes mellitus        | 2.00     | 0.000      |       |              |
| Prophylactic             | 2.00     | 0.000      |       |              |
| Diabetic nephropathy     | 1.83     | 0.167      |       |              |
| STZ + Caffeic acid       | 1.43     | 0.202      |       |              |
| Urea (mg/dl)             |          |            |       |              |
| control                  | 38.00    | 5.000      |       |              |
| placebo                  | 33.50**  | 2.500      | 72.806| 0.0001       |
| diabetes mellitus        | 57.33    | 2.231*     |       |              |
| Prophylactic             | 84.29    | 3.107*     |       |              |
| Diabetic nephropathy     | 81.67    | 1.498      |       |              |
| STZ + Caffeic acid       | 30.14    | 2.857*     |       |              |
| BUN (mg/dl)              |          |            |       |              |
| control                  | 17.50    | 2.500*     |       |              |
| placebo                  | 15.50*   | 1.500*     | 71.657| 0.0001       |
| diabetes mellitus        | 26.67    | 1.085*     |       |              |
| Prophylactic             | 39.29    | 1.392*     |       |              |
| Diabetic nephropathy     | 38.17    | 0.703      |       |              |
| STZ + Caffeic acid       | 25.86    | 1.010*     |       |              |
| HDL-cholesterol (mg/dl)  |          |            |       |              |
| control                  | 34.50    | 2.500      | 3.750 | 0.550        |
| placebo                  | 31.00    | 1.000      |       |              |
| diabetes mellitus        | 33.17    | 2.798      |       |              |
| Prophylactic             | 28.71    | 1.443*     |       |              |
| Diabetic nephropathy     | 28.17*   | 1.600      |       |              |
| STZ + Caffeic acid       | 25.86    | 1.010*     |       |              |
| Triglycerides (mg/dl)    |          |            |       |              |
| control                  | 134.00   | 7.000*     | 20.844| 0.0001       |
| placebo                  | 122.00   | 3.000*     |       |              |
| diabetes mellitus        | 180.83*  | 6.735*     |       |              |
| Prophylactic             | 50.71*   | 7.677*     |       |              |
| Diabetic nephropathy     | 51.83    | 17.252     |       |              |
| STZ + Caffeic acid       | 129.00   | 11.093*    |       |              |
| LDL-cholesterol (mg/dl)  |          |            |       |              |
| control                  | 20.00    | 14.000     | 2.786 | 0.040        |
| placebo                  | 28.00    | 5.000      |       |              |
| diabetes mellitus        | 11.33    | 11.910     |       |              |
| Prophylactic             | 61.71    | 12.124     |       |              |
| Diabetic nephropathy     | 26.33    | 7.186      |       |              |
| STZ + Caffeic acid       | 30.29    | 9.203      |       |              |
| Total Cholesterol (mg/dl)|          |            |       |              |
| control                  | 81.50    | 12.500     | 1.109 | 0.382        |
| placebo                  | 83.50    | 6.500      |       |              |
| diabetes mellitus        | 81.00    | 10.954     |       |              |
| Prophylactic             | 100.43   | 11.193     |       |              |
| Diabetic nephropathy     | 78.00    | 8.714      |       |              |
| STZ + Caffeic acid       | 71.71    | 7.402      |       |              |
| Urine creatinine Concentration (mg/dl) |          |            |       |              |
| control                  | 59.00    | 1.000      | 24.659| 0.0001       |
| placebo                  | 55.50    | 1.500      |       |              |
| diabetes mellitus        | 38.17    | 2.574      |       |              |
| Prophylactic             | 115.71   | 11.047     |       |              |
| Diabetic nephropathy     | 33.17    | 2.676      |       |              |
| STZ + Caffeic acid       | 60.43    | 0.948*     |       |              |
| Urine volume (liter/day) |          |            |       |              |
| Continued                |          |            |       |              |
in the tubular cells of the control and diabetic rats. However, an increased level of autophagy was observed in the groups treated with caffeic and prophylactic group. Autophagic vacuoles including autophagosomes and autophagolysosomes were markedly increased in the proximal tubular cells. Double membrane vacuoles containing electron-dense material, degenerating cytoplasmic organelles and cytosol, mitochondria with loss of visible cristae were frequently observed in the tubules.

### Effect of caffeic acid on the expression of autophagy transcripts in DKD.

At the end of the 12 weeks, **RB1CC1**, **ATG12**, **MAP1LC3B** mRNA expression were estimated in the kidney of the 6 groups and were downregulated in the DKD group compared to the naïve group (3.8, 5.2 and 13 fold, respectively).

Caffeic acid administration in the treated and prophylactic groups resulted in a significant induction of renal **RB1CC1**, **ATG12**, **MAP1LC3B** mRNA expression, compared to the control group \(F = 6.3, 4.9 \text{ and } 4.7\), respectively, at \(p < 0.01\)). However, we could not find any significant difference between the treated and prophylactic groups (Table S2 and Fig. 4a–c).

### Effect of caffeic acid on the renal expression of miR-133b, -342, 30a in DKD.

At the end of the 12 weeks, miR-133b, -342, 30a expression were estimated in the kidney of the 6 groups and were up-regulated in the DKD group compared to the naïve group (1900, 175 and 125 fold, respectively).

Caffeic acid administration in treated and prophylactic groups resulted in a significant down regulation of renal miR-133b, -342, 30a expression, compared to the control group \(F = 6.7, 7.896 \text{ and } 7.814\), respectively at \(p < 0.01\)). Notably, there was no significant difference between the treated and prophylactic groups (Table 3 and Fig. 5a–c). Of note, there was a significant negative correlation between the chosen DN-characteristic miRNAs and their corresponding autophagy target genes\(p < 0.01\) among all the groups of the study (Table 4).

### Discussion

Until the current study, whether caffeic acid exerted any curative or prophylactic effect in DN was unknown. To address this question, we investigated the effect of caffeic acid on urinary albumin excretion in HFD-STZ induced diabetic rats. Obesity was induced in rats with high-fat diet and streptozotocin injection, which affects islet \(\beta\)-cells and results in insulin resistance\(^{12, 13}\). Urinary albumin has been used as a predictive biomarker for prognosis of DN. Furthermore, the reduction in urinary albumin in DN reportedly were correlated with renal protection\(^{14}\). Herein, we show that DN caused significant increases in the urinary albuminuria, whereas caffeic acid suppressed this effect.

Caffeic acid treatment and prophylaxis markedly increased **RB1CC1**, **ATG12**, **MAP1LC3B** mRNA expression in diabetic kidneys and decreased congestion with marked vacuolation in diabetic glomeruli. We used transmission electron microscopy (TEM) to monitor the appearance of autophagosomes. As shown in Fig. 5A, no obvious autophagic vacuoles were found in control with numerous autophagic vacuoles appeared in caffeic acid treated and prophylactic group. In addition, Caffeic acid inhibited miR-133b, -342, 30a expression (epigenetic regulators of the chosen autophagy genes) in diabetic nephropathy rats. Our findings suggested that caffeic acid resulted in marked induction of autophagy in diabetic kidneys with potential curative and preventative effect. In agreement with previous studies that addressed the role of caffeic acid in improving glucose utilization in insulin-resistant

|                          | Mean  | Std. Error | F     | Significance |
|--------------------------|-------|------------|-------|-------------|
| Creatinine clearance     |       |            |       |             |
| control                  | 4.50  | 0.500\(^b\)| 138.861 | 0.0001      |
| placebo                  | 4.00  | 0.000\(^b\)| 33.400  | 0.0001      |
| diabetes mellitus        | 13.50 | 0.428\(^a\)|       |             |
| Prophylactic             | 17.71 | 0.747\(^a\)|       |             |
| Diabetic nephropathy     | 18.50 | 0.563      |       |             |
| STZ + Caffeic acid       | 4.00  | 0.218\(^a\)|       |             |
| Albuminuria              |       |            |       |             |
| control                  | 16.00 | 0.000\(^b\)| 135.014 | 0.0001      |
| placebo                  | 16.00 | 0.000\(^b\)|       |             |
| diabetes mellitus        | 28.17 | 0.872\(^a\)|       |             |
| Prophylactic             | 31.57 | 0.297\(^a\)|       |             |
| Diabetic nephropathy     | 35.17 | 0.749      |       |             |
| STZ + Caffeic acid       | 19.43 | 0.429\(^a\)|       |             |

Table 1. Effect of Caffeic acid on urine and serum metabolic and renal markers. \(^a\)\(p < 0.05\) compared to non diabetic control. \(^b\)\(p < 0.05\) compared to STZ induced DN control group.
Figure 2. Histopathological studies of kidney. (A) Control; (B) Naive; (C) Diabetes mellitus group; (D) Diabetic nephropathy group; (E) caffeic acid prophylactic group; (F) caffeic acid treated group. H&E X 400.

Figure 3. Transmission electron micrograph of renal tubules. (A) Control group: Normal structures of the nucleus, mitochondria with few scattered electron-dense lysosomes and intact bruch border were noted. (B and C) diabetic rats: accumulation of huge electron-dense lysosomes and few autophagic vacuoles were detected. (D and E) diabetic nephropathy group: apoptotic nucleus, partially degraded cytoplasm and elongated mitochondria. Also, double membrane of the autophagosome was clearly seen containing cytoplasmic debris. (F–H) treated diabetic rats: cytoplasm showed multiple scattered autophagosomes, autophagosomes with lysosomes (vacuoles containing electron dense material), and mitophagy (an autophagosome containing an undigested mitochondrion), indicative of autophagic activity. (I–K) diabetic prophylactic rats: numerous autophagosomes with degenerated membrane cellular debris and mitophagy L, lysosomes; N, nucleus; bb, bruch border; m, mitochondria; [↑], autophagosomes; [↑↑], autophagosomes with lysosomes; ▲▲, mitophagy.
mouse hepatocytes. Remarkably, caffeic acid phenethyl ester, a phytochemical extract of propolis was found to have antidiabetic effect and improve renal function tests in a rat model with renal tubular damage and oxidative

|                | Mean    | Std. Error | F     | Significance |
|----------------|---------|------------|-------|--------------|
| **ATG12**      |         |            |       |              |
| control        | 365.540000 | 91.7983929 | 4.960 | 0.001        |
| Nieve          | 187.699700 | 35.0997000 |       |              |
| DM             | 0.653364  | 0.1643958  | 4.460 | 0.001        |
| DN             | 0.518168  | 0.2456460  |       |              |
| Prophylactic   | 92.360886 | 46.6909842 |       |              |
| caffeic treatment | 253.094229 | 66.1801127 |       |              |
| **MAP1LC3**    |         |            |       |              |
| control        | 422.599900 | 60.8979453 | 4.797 | 0.001        |
| Nieve          | 236.678550 | 25.6485500 |       |              |
| DM             | 0.542120  | 0.0963318  | 4.797 | 0.001        |
| DN             | 0.511700  | 0.1984661  |       |              |
| Prophylactic   | 391.589014 | 60.9800118 |       |              |
| caffeic treatment | 492.281086 | 152.8860110 |       |              |
| **RB1CC1**     |         |            |       |              |
| control        | 39.311867 | 38.1394967 | 6.379 | 0.000        |
| Nieve          | 1.853000  | 0.7741000  |       |              |
| DM             | 0.313524  | 0.1130870  | 6.379 | 0.000        |
| DN             | 0.306620  | 0.1765692  |       |              |
| Prophylactic   | 286.109286 | 45.7528180 |       |              |
| caffeic treatment | 296.567486 | 54.8583762 |       |              |

Table 2. Differential Expression of Autophagy transcript among different groups of animal model.

Figure 4. **RB1CC1, ATG12, MAP1LC3B** expression in Kidney tissue at the end of the 12 weeks in the Control, Naive, Diabetes mellitus group, Diabetic nephropathy group, caffeic acid Prophylactic group, caffeic acid Treated group. (A) **RB1CC1 mRNA**; (B) **ATG12 mRNA**; (C) **MAP1LC3B**. Data are presented as fold change, where *indicates P < 0.05 compared to the control group (One way ANOVA followed by Bonferroni’s multiple comparison test), N = 8/group.
| miRNA  | Control | Nieve   | DM       | DN       | Prophylactic caffeic | Caffeic treatment |
|--------|---------|---------|----------|----------|----------------------|------------------|
| miRNA-133b | 0.857467 | 0.913150 | 1.239480 | 3.888120 | 0.225943              | 0.081271         |
|         | 0.268180 | 0.057850 | 0.378640 | 1.195533 | 0.1773867             | 0.0306317        |
|        | 6.769   | 0.0001  |          |          |                      |                  |
| miRNA-342 | 0.52057  | 0.83430  | 1.03610  | 4.28552  | 0.10760              | 0.06489          |
|         | 0.244219 | 0.344400 | 0.325210 | 1.253075 | 0.051428             | 0.019441         |
|        | 7.896   | 0.0001  |          |          |                      |                  |
| miRNA-30a  | 0.424800 | 0.701900 | 1.288600 | 6.355700 | 0.320486            | 0.310000         |
|          | 0.2929798 | 0.1481000 | 0.3440706 | 1.8278039 | 0.1147793          | 0.0891868        |
|        | 7.814   | 0.0001  |          |          |                      |                  |

Table 3. miRNA Expression among different groups of animal model.

Figure 5. miR-133b, -342, 30a expression in Kidney tissue at the end of the 12 weeks in the Control, naïve, Diabetes mellitus group, Diabetic nephropathy group, caffeic acid Prophylactic group, caffeic acid Treated group. (A) miR-133b; (B) miR-342; (C) miR-30a. Data are presented as fold change, where * indicates P < 0.05 compared to the control group (One way ANOVA followed by Bonferroni's multiple comparison test), N = 8/group.
Correlation between the selected miRNAs and autophagy transcript among the investigated groups of rats. **Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed). ***Indicates P < 0.001, Unpaired, two tailed t test, N = 8/group. r:Spearman correlation coefficient.

|                      | miRNA-133b | miRNA-342 | miRNA-30a | ATG12 | MAPLC3 | RB1CC1 |
|----------------------|------------|-----------|-----------|-------|--------|--------|
| miRNA-133b           | 1.000      | 0.893**   | 0.546***  | −0.483** | −0.425** | −0.543** |
| Sig. (2-tailed)      | 0.000      | 0.001     | 0.003     | 0.010  | 0.001  |
| miRNA-342            | 0.893**    | 1.000     | 0.685**   | −0.467** | −0.517** | −0.569** |
| Sig. (2-tailed)      | 0.000      | 0.000     | 0.004     | 0.001  | 0.000  |
| miRNA-30a            | 0.546***   | 0.685**   | 1.000     | −0.442** | −0.468** | −0.375** |
| Sig. (2-tailed)      | 0.001      | 0.000     | 0.007     | 0.024  |        |
| ATG12                | −0.483**   | −0.467**  | −0.442**  | 1.000  | 0.547** | 0.530** |
| Sig. (2-tailed)      | 0.003      | 0.004     | 0.007     | 0.001  | 0.000  |
| MAPLC3               | −0.425**   | −0.517**  | −0.468**  | 0.547** | 1.000  | 0.754** |
| Sig. (2-tailed)      | 0.010      | 0.001     | 0.004     | 0.001  | 0.000  |
| RB1CC1               | −0.543**   | −0.569**  | −0.375**  | 0.530** | 0.754** | 1.000  |
| Sig. (2-tailed)      | 0.001      | 0.000     | 0.024     | 0.001  | 0.000  |

Table 4. Correlation between the selected miRNAs and autophagy transcript among the investigated groups of rats. **Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed). ***Indicates P < 0.001, Unpaired, two tailed t test, N = 8/group. r:Spearman correlation coefficient.

stress16, 17. Moreover, previous studies explored the role of caffeic acid and its derivative in induction of autophagy10, 18.

Accumulating evidences indicate that change in the nutrient-sensing paths in diabetic states possibly will alter the autophagic response stimulated by cellular stress, which could subsequently result in diabetic nephropathy30. Impaired autophagy lead to accumulation of p62/SQSTM1 protein in proximal tubular cells30, activation of the mTOR pathway30 and inactivation of AMPK22. Modulation of the autophagy pathway has a great impact on development of a new nephro protective and therapeutic option23. Caffeic acid triggered induction of AMPK, class III PI3-kinase and the autophagic response in tumor11, 20.

Autophagy involves a series of dynamic membrane rearrangements controlled by a set of ATG proteins24. RB1-inducible coiled-coil protein 1 (RB1CC1 or ATG 17), which are needed for phagophore formation and initiation of autophagy25, 26. Microtubule-associated proteins 1A/1B light chain 3B is a central gene in the autophagy pathway where it functions in autophagosome membrane expansion and fusion events and have structural homology with ubiquitin27. ATG12, forms the ATG12–ATG5–ATG1 complex involved in autophagosome maturation28. Zahng et al., reported that, erlotinib increased renal autophagy, as indicated by altered expression and activity of ATG12, and LC3A II, in diabetic mice29. Fang and his colleagues demonstrated that the expression of autophagy related proteins such as Beclin-1, ATG12-ATG5 and LC3-II was markedly inhibited in DKD30. Recent evidence showed that induction of autophagy may be linked to maintaining renal homeostasis in diabetic kidney31. In the light of our results, it seems that caffeic acid might improve diabetic nephropathy through the restoration of autophagy activity in diabetic kidneys.

Because miRNAs are recently linked to regulation of autophagy pathway32, we applied combined bioinformatics analysis to retrieve DN related autophagy genes and their miRNA regulators. Accordingly, miR-133b, -342 and -30a target the above mentioned autophagy genes and were previously reported by our research group as urine markers for DN33. miR-30a inhibits autophagy by selectively down regulating ATG5 and Beclin 1 expression34. Targeting miR-30a, induces autophagy in response to imatinib treatment in chronic myeloid leukemia35, miR-30a and miR-34a play their key roles in the regulation of autophagy pathway such as PI3KCI/Akt/mTORC1 signaling pathway, and Ras-Raf-MAPK cascade36. Interestingly, miR-133b induced autophagic cell death in colon cancer cells37. Vitamin E-based therapy for hyperglycemia & T2DM triggers the expression of AMPK via regulation of miRNA-133b38. Moreover, miR-342-5p is coupled to the antiviral IFN response39 which in turn linked to autophagy.

Our Experimental model revealed that treatment with caffeic acid suppressed the expression of these miRNAs with subsequent induction of autophagy which ameliorated glomerular changes, albuminuria with reducing blood glucose levels in HFD-STZ-induced diabetic rats. We hypothesize that caffeic acid seems to trigger AMPK signaling, PIK3 pathway via regulation of miR-30a,-342,-133b which in turn induces autophagy that ameliorates diabetic nephropathy.

**Conclusion**

We adopt alternative strategies for better management of DN with the interest in the search of new drugs from natural sources and determining their mechanism of action which will be of great value in developing countries with limited resources and high incidence rates of diabetes mellitus. Also, there is a profitable concern in the
advancement of tools to suppress or stimulate miRNA expression associated with autophagy markers in vivo as an advance in the management of diabetes complications.

**Material and Methods**

**Animal Experiments.** All procedures for the care and use of laboratory animals were approved by the Institutional Animal Ethics Committee for Ain Shams University, Faculty of Medicine. All the methods were performed in accordance with the relevant Ethical guidelines and regulations (Ethical committee approval no FWA 000017585). Male Wistar rats (weighing 250–300 g) were purchased from National Research Institute (Cairo, Egypt) and were accommodated in an animal house with temperature (22 ± 1°C) and lighting (12 h light–dark cycle) control. Before the start of the experimental work, an adaptation one week during which rates were administered vehicle (tap water).

**Experimental protocol.** Caffeic acid and STZ were purchased from Sigma Aldrich. Forty-eight male Wistar rats weighing 250–300 g were divided into six groups (Fig. 6), 8 animals each as shown in Fig. 1. After one week adaptation, rats were split into a high-fat group (32 rats) which obtained a high-fat diet for four weeks and a normal age-matched control group (16 rats) which received a standard diet and subdivided into control and naïve groups. All high fat group after 4 weeks had received STZ 30 mg/kg i.v. once with high fat diet for another 4 weeks). HFD-STZ developed type II DM with fasting blood glucose above 16.7 mmol/L. Afterwards, they were haphazardly divided into two groups: diabetic control group (8 rats), DKD model group, 24 rats (They were further subdivided into three groups: diabetic nephropathy control group (8 rats); herbal extract caffeic acid (CA) treated group, 8 rats (CA 40 mg/kg body weight/day orally for 4 weeks) and pretreated group: 8 rats (received the herbal extract CA after induction of diabetes for 4 weeks). CA was dissolved in cold water and administrated
via intra-gastric gavage (i.g.) one time daily for twelve weeks according to Jayanthi et al.\(^40\) and Dhungyal et al.\(^41\) (Fig. 1). To study the effectiveness of CA at different doses in modulating renal function, creatinine clearance was estimated after CA treatment period in the HFD- STZ rats (Fig. 7).

**Induction of DKD.** Type 2 diabetes mellitus was provoked according to Zhang et al.\(^42,43\). In the normal group, rats were fed a standard chow diet of a total kcal value of 20 kJ/kg (52% carbohydrate, 20% protein, 5% fat), while diabetic group rates were fed a high-fat diet of a total kcal value of 40 kJ/kg (45% carbohydrate, 22% protein, 20% fat). The two groups were kept on their diets for 8 weeks duration. In the 4th week, a single low dose of STZ (30 mg/kg, dissolved in 0.1M sodium citrate buffer at pH 4.4) was injected into each rat of the diabetic group intraperitoneally.

After the STZ injection, rats obtained drinking water containing sucrose (15 g/L) for 48 hours, to reduce early death due to insulin discharge from partially injured pancreatic islets. Seventy two hours later, rats were checked for hyperglycemia and those with FBS more than 250 mg/dL were included in studies of diabetic nephropathy. Diabetic rats received long-acting insulin (2–4 U/rat) via S.C injection to maintain blood glucose levels in a desirable range (300 mg/dL) and also to prevent subsequent development of ketonuria\(^44\). Nephropathy was noted in rats (4–8 weeks) after the administration of STZ and was assessed in terms of significant increase in proteinuria, serum creatinine, blood urea nitrogen (BUN), extracellular matrix deposition and thickening of glomerular basement membrane. At the end of the eight-week pretreated and treated, rats were sacrificed using an intraperitoneal injection of sodium pentobarbital (50 mg kg\(^{-1}\)). Blood samples and kidney were collected for biochemical, histopathological analyses and TEM. A 24-h urine was collected on the day before scarification.

**Metabolic parameters, urinary albumin excretion, and renal function analysis.** Fasting blood sugar(FBG) was measured using the glucose oxidase method. Serum total cholesterol, LDL, HDL, Triglycerides, creatinine, and urine creatinine were detected by automated clinical chemistry analyzer (Olympus-2000, Tokyo, Japan). Samples from rat kidneys were snap frozen at −80°C for further RNA extraction, and another samples were processed for histopathological examination and TEM.

**Histopathological Examination.** Kidneys were fixed (10% neutral buffered formalin (NBF)), paraffin embedded, cut into 4μm sections and then stained with Hematoxylin and Eosin to be examined at 400 magnification by 2 independent histopathologists.

**Transmission electron microscopy (TEM).** Kidney tissue samples were fixed in glutaraldehyde and osmium tetroxide, washed with PBS& dehydrated. After exchange through acetone, the samples were later embedded in Epon 812. The kidney tissue were made into ultra-thin (70–80 nm) after observation and positioning, and were double stained following standard methods. All of the kidney samples were examined using TEM.

**Quantitative real-time PCR analysis for measurement of miRNAs and autophagy transcripts.** We have retrieved a set of 3 DN-characteristic miRNAs (miR-133b, miR-342 and miR-30a) based on previous microarray studies such as miro-Ontology database (available at [http://ferrolab.dmi.unict.it/miro/](http://ferrolab.dmi.unict.it/miro/)), miRWalk database distinguished DKD from other diseases. These 3 miRNAs were chosen related to diabetic nephropathy and targeting autophagy genes(RB 1-inducible coiled coil protein (RB1CC1), Microtubule-associated proteins 1A/1B light chain 3(MAP1LC3B), Autophagy related gene (ATG-12)).

Total RNA, including small RNA species, was extracted from kidney using mirNeasy\(^8\) Mini kit (Qiagen, Germany), according to the manufacturer’s recommendations. Total RNA concentration was measured by measuring absorbance at 260 nm using an Ultraspec 1000, UV/visible spectrophotometer (Amersham Pharmacia Biotech, Cambridge, England). Afterwards, A260/A280 and A260/A230 ratios were determined. A260/A280 ratio must be between 1.8 and 2.0. Afterwards, 500 ng total RNA from kidney was changed to cDNA by miScript II RT Kit (Qiagen, Valencia, CA) using miScript HiSpec buffer which was used in real-time PCR analysis using a miScript primer assay and the miScript SYBR Green Kit. The miScript Universal Primer (reverse primer) and QuantiTect SYBR Green PCR Master Mix, respectively were used as per manufacturer’s protocol to detect 3 miRs (miR-133b, miR-342, miR-30a) and 3 autophagy genes(RB1CC1, ATG12 and MAP1LC3B), respectively. RNA quality control and housekeeping gene (RNU6-2, GADPH) were also included in the assay for miRNA and autophagy gene expression, respectively. The qPCR tubes were run on a Step One Plus\(^8\) System (ABI). The primer of selected miRNAs, autophagy transcript and endogenous control were purchased from Qiagen. Data Analysis were done by using the ΔΔCT method of relative quantification\(^40\) on a Light-cycler, software v2.2.2 (StepOne™ Software). Data were presented as fold change in expression and were calculated as 2\(^{-\Delta\Delta CT}\). Where ΔCT = CT target gene − AVG CT reference gene and ΔΔCT = ΔCT (sample 2) − ΔCT (sample 1) where sample 1 is the control sample and sample 2 is the experimental sample.

**Statistical tests.** Data are presented as mean ± S.D. Statistical differences between the groups were estimated by one-way analysis of variance (ANOVA) followed by Bonferroni’s post-tests. A p-Value < 0.05 was considered significant. Statistical analyses carried out using Graph Pad Prism (GraphPad software) and with SPSS version 21.0 (SPSS, Chicago, IL, USA).

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Author Contributions
Matboli M: performed bioinformatics analysis, practical work, participated in the design of the study, statistical analysis and drafting the manuscript. Eissa S.: participated in the design of the study, data analysis, drafting and revising the manuscript, and has given final approval of the version to be published. Doaa Ibrahim: practical work and statistical analysis. Marwa Hegazy: Drafting the manuscript, data analysis. Imam S: choice of phytochemical extract, Drafting the manuscript. Eman K Habib: TEM examination.

Additional Information
Competing Interests: The authors declare that they have no competing interests.

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