SYZYGIUM AROMATICUM AMELIORATES OXIDATIVE STRESS AND FIBROSIS IN ADENINE-INDUCED CHRONIC KIDNEY DISEASE IN RATS

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ABSTRACT. Chronic kidney disease (CKD) is a slowly progressive disease. The prevalence and incidence of CKD are increasing, making it a worldwide public health problem causing a high economic burden to poor outcomes countries. The clinical complications of CKD include not only cardiovascular diseases (CVDs) but also renal anemia, renal failure and renal osteodystrophy. Here, we assessed the effect of treatment with clove extract (CE) on kidney structure and function using a chronic kidney disease experimental model induced by adenine. Twenty four rats were randomly divided into four groups: control group; clove extract (CE only) group; CKD (adenine only) group and CKD/clove extract (adenine treated with CE) group. After adenine and/or clove extract were given orally to rats for 4 weeks, kidney specimens and blood were collected for further histopathological examination and biochemical analyses. Results showed that adenine treatment caused serious renal pathological damages and increase of blood creatinine, urea nitrogen and phosphorus levels. However, the activities of renal antioxidant enzymes like superoxide dismutase (SOD) and glutathione peroxidase (GPX) were suppressed and the level of malondialdehyde was increased by administration of adenine. In addition, the expressions antioxidative transcription factor as Nrf2, heme-oxygenase1 (HO1) and endothelial nitric oxide synthetase (e-NOS) genes were down regulated. Meanwhile, the expression of Ki67, wnt7a, collagen IV and β-catenin genes were up regulated. However, these changes could be significantly reversed by the treatment with of clove extract (300 mg/kg), indicating clove extract could improve the kidney function of CKD via enhancing antioxidant ability and inhibiting inflammation and fibrosis. Clove extract markedly ameliorated renal histopathological markers of inflammation and fibrosis. In conclusion, Clove extract exhibits ameliorative effects against adenine-induced CKD in rats by reducing oxidative stress and inflammation and modulation of different molecular mechanisms.

Keywords: Chronic kidney disease, Adenine, Clove extract, Nrf2, Wnt7a, fibrosis

INTRODUCTION

Chronic kidney disease (CKD) is a global health problem that affects about 10% of the people in the world [1, 2]. CKD is considered as 12th leading cause of death in the world [3]. CKD affects both the kidney function and structure leading to tubular atrophy, cysts, changes in quantity and/or quality of urine and blood. This can be recognized by the increased concentrations of serum creatinine, blood urea nitrogen, uric acid, urine albumin in addition
to renal tubulointerstitial fibrosis as a last common character of CKD promoting the sequent loss of the function of the kidney [4].

Until now, there is no drug to ameliorate kidney function in patients with CKD and reduce disease progression. The recent therapeutic ways to decrease the disease progression are limited to the normalization of insulin, glucose and blood pressure [5]. Patients with CKD are at risk of progressing to end-stage renal disease (ESRD), which requires either dialysis or kidney transplantation [6].

Oxidative stress is a well-known risk factor for the evolution of many complications of CKD [7]. Subsequently, clinical medication interested only with early repression of the leading cause of kidney injury to keep the residue or delay chronic renal failure [8]. The establishment of a new medication is highly required to either slow or invert the decrease in the function of the kidney with particular interest in natural products with antioxidant activity and safety profiles [9]. Moreover, they might avoid the development of renal injury to end-stage renal disease (ESRD) or cardiovascular disease, where only transplantation or dialysis will be the only method of treatment. Both choices may be costly or unavailable in a lot of developing countries [10].

About 80% of the world’s population use Complementary and alternative medicines (CAMs) which became an important component of most parts of the health care system. Treatment of CKD with herbs has become a fruitful area for the entry of complementary medicine as one of the axes of treatments which may have more favorable safety profiles over the past decade [11].

Clove (Syzygium aromaticum) is considered as one of the most important spices that has been used for many medicinal purposes. Many reports explored the antioxidant, anti-inflammatory, antibacterial, antifungal, antiviral and anticancer activities of different spice plants especially Clove. It had attracted the attention due to its potent antioxidant and antimicrobial activities among the other spices [13]. Clove extract is used in the treatment of many health conditions, including cancer, migraine headaches, intestinal parasites, colds, asthma, stress and gastrointestinal problems such as diarrhea, gas and vomiting [14, 15].

Therefore, the critical role of oxidative stress and inflammation in the progression of the kidney disease [16, 17] and the reported protective effects of clove extract in different diseases [15] has been focused. Thus, the present study was designed to investigate the effect of clove extract on the biochemical, molecular and histopathological changes associated with chronic kidney disease using adenine induced rat model.

**MATERIALS AND METHODS**

**Chemicals**

Adenine purchased from Alfa-Aeser Co. Dried Plant clove (Syzygium aromaticum (L.) Merr.) purchased from a local market, Egypt. Kits for measuring urea, creatinine, phosphorus, malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx) were purchased from Biodiagnostic, Egypt.
Animals

Twenty-four male Wistar rats with weight range from 100g to 120 g were purchased from Theodore Bilharz Research Institute, Giza, Egypt. They were kept for one week for acclimatization having free access to a feed composed of standard powder diet and water. The current study was approved by Faculty of Science, Mansoura University, 2018. Animals were treated in accordance to Guide of the Care and Use of Laboratory Animals, Faculty of Science, Zoology Department, Mansoura University, 2018.

Plant extraction

Clove plant powder was extracted with methanol (80%) for six days with continuous agitation. After filtration of the mixture, it was allowed to dry in the air. Then, the dried extract was stored at -20 °C till use.

Experimental design

After seven days as an acclimatization period, all rats were separated into four equal groups (6 rats each) as following: the first group (control group): this group allowed to have the same diet with no additions till the end of the study. The second group (normal treated group): this group was given clove extract orally by gavage for four weeks with a dose of 300 mg/kg/day. The third group (CKD group): this group was shifted to a diet with adenine with a dose of 0.75% w/w in feed daily for 4 weeks. The fourth group (CKD treated group): this group was treated with adenine like the third group for four weeks then treated with clove extract orally for another four weeks. After 24 hours of clove extract treatment, the animals were sacrificed by decapitation. Blood specimens were collected and kept for 30 min to clot. Serum samples were then obtained by centrifugation for 10 minutes at 3000 g and kept at -20°C. The kidney was immediately dissected out, washed with ice-cold saline, blotted dry and kept for measuring different biochemical parameters.

Preparation of homogenates

An accurate weight from the kidney tissue homogenized in phosphate-buffered saline solution with pH 7.4 using a Teflon pestle attached to a homogenizer motor. The homogenate was diluted to 5% (w/v). The homogenate was centrifuged at 4000 g at 4°C for 20 minutes to remove cell debris and nuclei. The obtained supernatant were utilized for biochemical analyses.

Biochemical measurements

Creatinine, urea and phosphorous were measured spectrophotometrically in serum using biodiagnostic kits. The oxidative stress parameters (SOD, GPx and MDA) were measured in the kidney homogenate supernatant spectrophotometrically using a commercial kit (Biodiagnostic, Egypt).
Real-time Polymerase Chain Reaction

RNA extraction

Kidney samples were deposited in RNeater™ solution (cat no. AM7024, life technologies). Then it was kept at -80 °C. The samples were handled by the TRizol™ reagent technique (catno15596026 Invitrogen). The assay was done according to the protocol assumed by the manufacturer. In brief, 1 ml of TRizol™ Reagent added on each 0.1g of tissue followed by homogenization. RNA was extracted from every homogenate by the addition of 200 μl chloroform per 1 ml of the TRizol™ Reagent used for lysis, then securely cap the tube. The tubes were mixed and Centrifuged for 15 minutes at 12,000 × g at 4°C. The mix was separated into the phase of red phenol-chloroform at the bottom and a colorless hydrous phase at the top. The hydrous phase with the RNA was transferred to a clean tube. All RNA precipitate forms a pellet-like white gel at the bottom of the tube. The supernatant was decanted carefully. The samples were mixed by vortex for 15 seconds, followed by incubation at room temperature for two minutes, then centrifuged for five minutes at 7500 x g at 4°C. RNA pellet was dried at room temperature for five to ten mins. The pellet was suspended in 25 μl of RNase-free water. Finally, Incubated at 55–60°C for 10–15 minutes.

Synthesis of complementary DNA (cDNA)

Complementary DNA (cDNA) has been synthesized using 2X Reverse Transcription (RT) Master Mix kit (Archive, High Capacity cDNA Reverse Transcription Kit). Briefly, the protocol was to add 10 μl of RNA sample to 10μl of RT-pcr master mix, 1 μl MultiScribe™ Reverse Transcriptase, 2μl Primers, 1 μl RNase Inhibitor and 4.2 μl nuclease-free H₂O) then stand for 120 minutes at 37°C. Then store it at -80°C.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

The mRNA expression of collagen IV, Wnt7a, eNOS, Nrf2, β-catenin and HO-1 were determined using qRT-PCR by mixing cDNA with 2X SYBR Green Master mix (cat no:K0251,Thermo Scientific Maxima SYBR Green qPCR Master Mix (2X)) and primer pairs (Table 1), qRT-PCR was done as following: initial denaturation at 95 °C for ten minutes, 40 cycles of denaturation for 15 seconds at 95 °C, annealing for thirty seconds, and extension for thirty seconds at 72 °C. the results was obtained by step one plus Applied Biosystem by the following equation [18]: $2^{-\Delta\Delta C_T}$
Table 1. The primer sequences for the tested genes:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen IV</td>
<td>F:5-ATAGAGAGAAAGCGGATGTCAAGA-3</td>
</tr>
<tr>
<td></td>
<td>R:5-GGATATAATTCTAGGTTGCTGCT-3</td>
</tr>
<tr>
<td>Wnt7a</td>
<td>F:5-GCCCACCTTCTGAGATCAAAG-3</td>
</tr>
<tr>
<td></td>
<td>R:5-TGGGTCTCTTCAAGATAATTG-3</td>
</tr>
<tr>
<td>β-catenin</td>
<td>F:5-TGAAGGTGCTGTCTGCTGCTC-3</td>
</tr>
<tr>
<td></td>
<td>R:5-TGCATCGGACCAGTTTTCAGA-3</td>
</tr>
<tr>
<td>eNOS</td>
<td>F: 5-GGACCCAAGTTTCTCGAGTA-3</td>
</tr>
<tr>
<td></td>
<td>R: 5-GGATCCAAAGCAGCTCTT-3</td>
</tr>
<tr>
<td>Nrf2</td>
<td>F: 5′-ATTGCTGTCATCTGTCAG-3</td>
</tr>
<tr>
<td></td>
<td>R: 5′-GCTATTTTCCATCCCGAGTTAC-3</td>
</tr>
<tr>
<td>HO-1</td>
<td>F: 5′-TGCTTTGCAGCTCTTCAGTTAC-3</td>
</tr>
<tr>
<td></td>
<td>R: 5′-CTTTCAGAAGGGTGAGGTAC-3</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5′-TATCGGACGCCTGGTTAC-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-CTGTCGCGTGAACCTTG-3′</td>
</tr>
</tbody>
</table>

Immunohistochemical investigations:

Ki-67 expression was assessed through the immunostaining of deparaffinized samples slides using particular horseradish peroxidase (HRP) conjugated antibodies. Ki-67 immunostaining was implemented via rabbit polyclonal antibody, (Cat. No. RB-9043-p, with dilution 1:300) Thermo Fisher Scientific, USA. Positive Ki-67 nuclei were counted under 200 magnification at 20 fields in every single group.

Histopathological investigations:

Kidney samples were fixed in 10% neutral buffer formalin, dehydrated in ethanol then cleared in xylene before being settled down into a melted paraplast. 5mm thickness section slides were stained routinely using hematoxylin and eosin (H&E) stain procedure and for further histochemical investigation of interstitial fibrosis and tubular atrophy so Masson trichrome (MT) and periodic acid-Schiff (PAS) and histochemical stains were performed respectively following the standard protocols. All specimens were then mounted for further microscopic examination. Random images were picked out at 200x magnification and then five scopes were pictured. Consistent with previous studies, tubular atrophy was assessed using semiquantitative scoring assigning each section as a score of renal tubular damage on a scale of grade 0-4: Grade 0, no damage; grade 1, interstitial fibrosis < 25% and mild tubular atrophy of the cortical space; grade 2, interstitial fibrosis 25-50% and moderate tubular atrophy of the cortical space; grade 3, interstitial fibrosis 50-75% and severe tubular atrophy of the cortical space; grade 4, interstitial fibrosis >75% and entire tubular atrophy of the cortical space [19, 20].
Statistical analysis

All data are introduced as mean ± S.E.M., SPSS version 14 (SPSS, Chicago, IL, USA) was applied to obtain the statistical analysis. One-way analysis of variance (ANOVA) test was utilized to study the statistical significance of the parameters among groups with a significance value P-value < 0.05.

RESULTS

Biochemical measurements

The kidney function in adenine treated group (0.75% w/w for four weeks) was characterized by significant elevation in the creatinine, Phosphorus in serum and BUN (P< 0.001). Treatment with CE reduce the effect of adenine and improve the renal function compared to the corresponding values of control group (Table 2).

Table 2. The effects of clove extract and/or adenine on the kidney function tests (creatinine (mg/dl), blood urea nitrogen (mg/l) and phosphorus (mg/dl) in serum of different groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal control</th>
<th>Normal treated</th>
<th>CKD</th>
<th>CKD treated CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine</td>
<td>1.06 ± 0.027</td>
<td>1.09 ± 0.015 NS</td>
<td>2.40 ± 0.05 #</td>
<td>1.20 ± 0.02 **</td>
</tr>
<tr>
<td>Blood urea nitrogen</td>
<td>12.14 ± 1.89</td>
<td>13.17 ± 1.426 NS</td>
<td>44.02 ± 3.52 #</td>
<td>27.125 ± 1.678 **</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>6.77 ± 0.34</td>
<td>6.72 ± 0.2 NS</td>
<td>9.03 ± 1.015 #</td>
<td>7.08 ± 0.22 **</td>
</tr>
</tbody>
</table>

All values were shown as mean ± SEM. One-way ANOVA with P≤ 0.05 considered as significant. # Significant compared to normal control group, **Significant compared to CKD group and NS Not significant compared to normal control group.

Oxidative stress and Antioxidant Markers

Adenine treated rats caused a significant elevation in the renal content of MDA (a lipid peroxidation marker) and a significant decrease in the activity of renal GPx and SOD enzymes after comparing with that values of normal control group (p < 0.05). Treatment with CE caused a significant reduction in MDA and a significant elevation in GPx and SOD activity compared to CKD group (p < 0.05) (Table 3).
Table 3. The effects of clove extract on the activities of glutathione peroxidase (GPx, u/gm tissue), superoxide dismutase (SOD, U/g tissue) and malondialdehyde levels (MDA, nmol/ml) in the renal of different groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal control</th>
<th>Normal treated</th>
<th>CKD</th>
<th>CKD treated CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPX activity</td>
<td>76.25 ± 8.17</td>
<td>102.18 ± 11.80</td>
<td>48.63 ± 4.35*</td>
<td>71.97 ± 4.13**</td>
</tr>
<tr>
<td>MDA content</td>
<td>1688.4 ±106.15</td>
<td>1427.45 ± 193.87 NS</td>
<td>2987.57 ± 312.04*</td>
<td>2173.9 ± 273.25**</td>
</tr>
<tr>
<td>SOD activity</td>
<td>606.21 ± 6.64</td>
<td>616.18 ± 7.94 NS</td>
<td>302.54 ± 6.70*</td>
<td>520.95 ± 8.59**</td>
</tr>
</tbody>
</table>

All values were shown as mean ± SEM. with p≤ 0.05 considered as significant. *Significant compared to normal control group, **Significant compared to CKD group and NS Not significant compared to normal control group.

Gene expression profiles

Adenine administration caused highly significant increase in the mean expression of collagen IV, Wnt7a and β-Catenin in the kidneys of the CKD group rats by comparing to normal control group (P< 0.001). In contrast, the treatment with clove extract showed a significant decrease in the mean expression of kidney collagen IV, Wnt7a and β-Catenin in the CKD-clove extract treated group when compared to CKD group (P< 0.001) (Fig. 1A-C). Renal mRNA expression of Nrf2, Ho-1 and e-NOS significantly down-regulated by adenine in the CKD group compared to the normal control group (P< 0.001). After the treatment with CE in the CKD treated group, there was a highly significant elevation in their expression when compared to the CKD group (P< 0.001) (Fig. 2A-C).
Fig. 1. The effect of clove extract on the expression of β-Catenin(A), collagen IV (B) and Wnt7a (C) genes in the kidneys of rats in different groups. Each chart showed mean ± SEM (n=6). The differences among all groups have been assessed by one-way ANOVA with (P < 0.05) is considered significant.
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**Fig. 2.** The effect of clove extract on the expression of the genes e-NOS (A), Ho-1 (B) and Nrf2 (C) in the kidneys of rats in different groups. Each chart showed mean ± SEM (n=6). The differences among all groups have been obtained by one-way ANOVA with (P < 0.05) considered as significant.

**Immunohistochemical investigation**

Both Clove extract and control groups showed almost negative Ki-67 expression in glomeruli and tubular cells (have brown-colored nuclei) in the cortex zone of renal tissues indicating slight evidence of proliferation. Whereas, in adenine treated group, showed positive cells within renal glomeruli and tubular cells as well represented marked expression of Ki-67 protein referring to the incidence of proliferation profile within renal cells. In contrast to the previous group, the kidney samples of the group of CKD treated with clove...
extract displayed weak expression for Ki-67 antigen referring to a significant drop in the proliferation profile (Fig. 3: A).

**Histopathological investigations**

Effects of adenine and/or clove extract on the kidney of rats were histopathologically examined and the results were displayed in (Fig. 3: B, C, D). The control group showed undamaged and normal kidney while the group treated with adenine showed remarkable histological changes with renal tubular degeneration, indicating obvious kidney damage. However, such palpable kidney damage is less remarkable with clove extract treatment as demonstrated by the following observations:

**a- Haematoxylin and Eosin**

There are no pathological alterations noticed within both previously mentioned groups. However, by the administration of rats by adenine showed intense signs of lesions, such as glomerular atrophy, expansion of the renal space of the glomerular capsules, interstitial congestion, clear necrosis in epithelial cells within renal tubules and pyknotic nuclei. On the other hand, marked recovery of most of the renal architecture and even histology except the occurrence of slight widening in the renal space of the glomerular capsules are appeared in the adenine treated clove buds extract group (Fig. 3: D).

**b- Periodic acid-Schiff (PAS) stain**

This study considered PAS stain in order to assess the extent of glycogen within different investigated groups and the impact of clove buds extract on CKD induced by adenine histochemically. In case of PAS stain, there were evident amelioration signs within adenine and clove buds extract treated rats compared to adenine treated ones that displayed widespread of mesangial mass in adenine treated group with marked glycogen depletion along with dilated renal tubules, which conversely were scarcely noticed in adenine and clove buds extract treated group. Control and/or clove extract-treated groups showed intact standard glycogen appearance within samples (Fig. 3 C).

**c- Masson trichrome stain**

Results of Masson trichrome stain revealed the intense propagation of the interstitial fibrosis within examined renal tissues of adenine treated group whereas, fibrosis was barely seen in clove and adenine treated group indicating the improvement influence of Clove extract on renal tissues. Alternatively, Control and/or clove buds extract-treated groups exhibited weak reaction for fibers existence, as showed in Fig.3: B. These histological results mentioned above were also confirmed by the statistical charts as showed in Fig. 4.
Fig. 3. Morphological changes in the kidney of studied groups stained with: A: Immunostaining for Ki-67: Ki-67 expression in renal tissue (a,b) exhibited weak reaction, (c) exhibited marked positive reaction, and (d) moderate to weak reaction. B: Masson Trichrome (MT) stain displayed collagen deposition (a,b) exhibited normal appearance of glomeruli and intact Bowman’s capsule, (c) displayed renal tissue with necrotic and swollen tubular cells intense deposition of collagen fibers (blue color), (d) displayed diminution of collagen deposition. C: Periodic acid-Schiff (PAS) stain displayed glycogen deposition (magenta-purple color) within cortex zone of different kidney treated samples (a,b) displayed normal glycogen deposition, (c) displayed massive depletion in glycogen deposition in addition to tubular atrophy, (d) group displayed a reduction in both glycogen depletion and tubular degeneration. D: Images of H&E stained cortex zone of various kidney samples (a,b) revealed the typical structure of this layer (G) glomerulus, (Pt) proximal tubule, (Dt) distal tubule, and (arrow) reasonable renal space within the renal capsule, (c) revealed massive degenerations (G) glomerular atrophy with focal segmental glomerulosclerosis, (arrow) expansion of the renal space of the glomerular capsules, (white curved arrow) interstitial congestion, (*) necrosis, and (arrowhead) pyknotic nuclei, (d) revealed marked improvement with (arrow) slight widening in renal space.
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**DISCUSSION**

Chronic kidney disease (CKD) was considered to be one of the major rising health troubles in developing countries. Nowadays, several plant parts such as leaves, roots, or even buds have been beneficial in a plethora of challenging diseases. For that, this study explored the probable impact of clove buds extract on adenine induced chronic kidney diseases.

Our results showed the treatment of chemically induced of CKD using adenine with clove extract ameliorated most of the adverse effects. CKD can be induced by adenine and its metabolite, 2,8-dihydroxyadenine, which are slightly soluble hence precipitate in the renal tubules causing their obstruction [21], leading to oxidative stress and therefore renal injury [22]. Adenine induced many physiological and biochemical abnormalities indicating kidney injury [23]. It caused renal dysfunction showed in increased serum BUN, creatinine and elevated the concentration of phosphorus which can be considered as a uremic toxin as it contributes to several metabolic disturbances which are in agreement with previous studies [23, 24], this study showed significant improvement of the increased concentration of serum creatinine, BUN and hyperphosphatemia that caused by adenine by clove extract treatment.

**Fig. 4.** Representative charts displaying variance between groups where:
A: labeling index of KI-67, B: the interstitial fibrosis score, C: the score of tubular atrophy and D: average of the glomeruli area in kidney of adenine and/or clove extract treated rats. (* ) significant compared to control group, (#) significant compared to adenine treated group
This may be referred to as an amelioration in the tubular and glomerular functions by clove extract.

Oxidative stress plays a significant role in the progression of CKD and its complications such as interstitial fibrosis, hypertension, uremia and anemia[25, 26]. The present study showed a state of increased oxidative stress in kidney tissues indicated by a decrease in the antioxidants as GPx and SOD enzymes and elevate the level of MDA (a marker of lipid peroxidation). Some investigators demonstrated that the treatment with adenine caused a significant increase in the markers of oxidative stress in the kidney [5]. On the other hand, the treatment with clove extract showed a significant improvement in the signs of oxidative stress in kidney tissues of rats that treated firstly with adenine supporting our hypothesis that the basis of the palliative action of clove on adenine-induced CKD is most likely the antioxidant action of clove extract.

Under normal conditions, oxidative stress stimulates endogenous antioxidants, enzymes and cytoprotective proteins that limit or inhibit tissue damage and dysfunction. Nuclear factor-erythroid-2-related factor 2 (Nrf2), a master organizer of the cellular antioxidant response that act by the following mechanism: when there is an increase in cellular oxidative stress, it is separated from Kelch-like ECH-associating protein 1 (Keap1) and then translocates to the nucleus resulting in expression of hundreds of genes [27], most of them encoding for antioxidant/detoxifying enzymes like the induction of direct antioxidant (SOD, Gpx and GSH ) and phase II enzymes like heme oxygenase-1 (HO-1) [28].

The present study showed a decrease in Nrf2 expression and hence of its downstream genes in the kidney tissue of adenine treated rats and this agrees with that reported in [29], [30]. However, the existence of inflammation and oxidative stress, that should have increased Nrf2 expression, this may be to contribute to the pathogenesis of oxidative stress and to amplification of its harmful effects on the kidney [29] this can be considered as a cause of the decrease in SOD and GPx levels as they are of the important genes induced by the transcription factor Nrf2 and these findings agree with that reported in [32]. Moreover the treatment with clove extract (300 mg/kg/day) significantly up-regulated the Nrf2 expression in the kidney; this indicates that clove extract has the ability to elevate the expression of Nrf2 gene, which suggest antioxidant activity and renoprotective effect of clove extract.

Heme oxygenase 1 (HO-1) is highly inducible upon stressful conditions and inflammation, it is generally known that HO-1 has physiological roles in cytoprotection, modulation of the inflammatory response and antioxidative functions owing to the effect of degradation of heme which has severe toxic effects on the kidney and other organs, and act as a pro-inflammatory activity and as a result of the inflammation induced by the pathology of renal failure [33]. HO-1 is the main factor responsible for the heme degradation giving rise to the formation of carbon monoxide (CO), ferrous iron (Fe++) and biliverdin (BV) which is rapidly reduced to bilirubin (BR) by biliverdin reductase [34]. The decrease in Nrf2 gene expression should have followed by a decrease in its downstream genes like HO-1 gene expression. So, our study, demonstrated that, the adenine treated rats showed a significant reduction in expression of the HO-1 confirming the oxidative stress induced by adenine feeding for four weeks. On the other hand, the treatment with clove extract (300 mg/kg/day) significantly increased the expression of HO-1 gene in the kidney of the rats confirming the antioxidant activity of clove extract.
Additionally, the present study showed a decrease in endothelial nitric oxide synthase enzyme expression (eNOS) in kidneys of CKD group indicating that vasodilator responses of the vascular endothelium were impaired by adenine as eNOS enhances the production of Nitric Oxide which has vasodilating and anti-inflammatory activities [30]. The treatment with clove extract resulted in a significant increase in the kidneys expression of eNOS in adenine treated rats suggesting that the increase in the expression of eNOS might be another possible renoprotective role for clove extract against CKD induced by adenine.

The canonical Wnt/β-catenin pathway has an essential role in nephrogenesis [31]. Wnt signals are expressed with a low level in adult kidneys at normal conditions hence it is activated again in kidney diseases [12]. In our study, adenine induced the expression of Wnt7a and its downstream gene β-catenin in the kidney moreover treating the rats with clove extract reduced the increased expression of Wnt7a and β-catenin mRNAs and these findings suggest that clove extract improve CKD induced by adenine through the regulation the Wnt/β-catenin signaling pathway.

Recently, Wnt signaling pathway has been shown to have a significant role in renal fibrosis by the activation of its target genes that result in synthesis of extracellular matrix constituents like Collagen IV. Renal fibrosis as a final pathological face of CKD [36]. There is a direct and positive relationship between the stage of tubulointerstitial fibrosis and the reduction in renal function in this disease [31]. In the current study, clove extract decreased adenine-induced renal overexpression of Collagen IV gene and this indicates that clove extract has a role in the amelioration of renal fibrosis.

It would be also interesting to Ki-67 antigen that is utilized as a nuclear protein marker of proliferation, besides it is well known to be expressed within all cell cycle stages except resting phase G0 [37]. Our findings revealed major expression of Ki-67 antigen in adenine administered rats, that was in agreement with Terada et al (2018). They reported that positive response of regenerating epithelial cells in acute injury induced kidney samples when stained with β-catenin, a multifunctional protein which plays a crucial role in renal damage and the regeneration of renal tissue as well, showed Ki-67 positivity likewise referring to active proliferation occurrence [38]. So, these findings presumed that β-catenin expression in damaged renal epithelial cells could be used for the detection of EMT incidence during the development of renal fibrosis. In the present study the cytological alterations demonstrated by light microscopy of kidney, obtained from adenine treated group indicated that the kidney of rats showed severe pathological damage such as tubular atrophy, interstitial fibrosis and increasing the thickness of Bowman capsule’s. These results was supported by previous observations of [32, 39, 40]. While these finding were ameliorated by treatment adenine-treated group with Clove buds extract and these observations are supported by study of [41, 42].

Another interesting finding was that, the renal dysfunction mediated by adenine is a sign of CKD and more detailed histological investigation indicated the precipitation of 2,8-DHA, interstitial mononuclear inflammation, tubular degeneration, etc. (Fig. 3). These results thus show that accumulation of adenine crystals in the kidney may trigger renal cell inflammation, renal tubular degeneration, renal dysfunction and at the end lead to CKD. Such histopathological changes were minimized after treatment with clove extract suggesting its effective protection and amelioration the kidneys from adenine effect.
CONCLUSION

This study indicates that clove extract ameliorated adenine induced chronic kidney disease model in rats by the reduction of renal oxidative stress and different molecular mechanisms owing to its renoprotective effect that include the induction of NRF2, HO-1 and eNOS pathways and its role in reducing the renal fibrosis by the suppression of wnt-β catenin pathway and the reduction in collagen IV and Ki67 expression. In addition, clove extract declined the increased proliferation rate caused by adenine in the kidney. Moreover, the effect to abate the histological effects induced by the adenine. These findings identify clove extract as a promising natural extract to avoid the progression chronic renal disease and may offer a new candidate to prevent or palliate CKD.

REFERENCES


