The Possible Protective Effect of Chlorophyllin and Vitamin D3 on Non-steroidal Anti-Inflammatory Drug Induced Renal Injury in Adult Albino Rat

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ABSTRACT
Background: Analgesic nephropathy is a renal disease which was characterized by papillary necrosis and chronic interstitial nephritis and was occurred by long-term consumption of analgesic factors. Analgesic nephropathy is one of the more common causes of chronic kidney disease Aim of the Work: Studying the possible protective effect of chlorophyllin and vitamin D3 on diclofenac induced renal injury in albino rat.

Material and Methods: 32 males (adult albino rat) divided to 4 groups: I (control), II (diclofenac as treatment): received daily intramuscular (IM) injection of 4 mg/kg diclofenac for 15 days, III (vitamin D3 as treatment): received oral daily dose of vitamin D3 (1000 IU/kg) for 15 days before diclofenac injection and continued for another 15 days, IV (chlorophyllin as treatment): received daily intraperitoneal (IP) injection of 30 mg/kg chlorophyllin similar to aforementioned way in group III. Biochemical studies were performed to assess urea and creatinine, NADPH oxidase, Superoxide dismutase and IL-2, MDA, TNF-α by ELISA. Quantitative assessment of Caspase-3 and Nrf2 gene expression were also performed by real-time PCR. Histological, histochemical and immunohistochemical investigations were done. Morphometric measurements of optical density of PAS reactions and Ki67 immunoexpressive cells area % were done. All measurements were followed by statistical analysis.

Results: Diclofenac only treated group showed marked distortion in glomeruli and renal tubules. Significant decrease in PAS reaction (optical densities), with decrease in mean number of Ki67 immunoexpressed cells. These histological changes were accompanied by alterations in biochemical measurements. Pretreatment with chlorophyllin and vitamin D3 significantly improved the histological and biochemical changes.

Conclusion: A protective effects of chlorophyllin and vitamin D3 were found in diclofenac-induced cortical renal damage. This evidenced through reversing biochemical and pathological change in rat.

INTRODUCTON
Analgesic nephropathy is renal disease which was characterized by papillary necrosis and chronic interstitial nephritis and caused by long-term consumption of analgesic factors. Analgesic nephropathy is one of the more common causes of chronic kidney disease (CKD). Diclofenac sodium (Voltaren) is a non-steroid anti-inflammatory, with a potent anti-inflammatory, analgesic, and antipyretic activity. Its therapeutic effect is achieved by inhibiting various types of prostaglandin. This is mediated via inhibition of the cyclooxygenase (COX) enzyme, reducing the arachidonic acid release, and arachidonic acid uptake. Antinociceptive effect of diclofenac may result from the activation of some potassium channels [1]. As results to extensively using to analgesic and anti-inflammatory agents, toxicities occur many times especially when pain therapies, inflammation and fever used with high doses for long period. The common organs involved are liver, kidney and GIT (2). Diclofenac is commonly associated with aminotransferase elevations. The exact mechanism for hepatotoxicity with diclofenac is unknown till now but is thought to multifactorial as might be caused by mitochondrial impairment, cellular injury by binding to cell protein and consumption of NADPH (3,4). Toxic effects on GIT include serious upper GIT complications as peptic ulcer perforations, obstructions and bleeding (5). Kidney is the target organ for the adverse effects of Diclofenac. The drug is metabolized by the liver into 4-hydroxy diclofenac and other hydroxylated forms, and then undergoes glucuronidation or sulfation followed by biliary and urinary excretion. It affects renal functions through their action on renal prostaglandins (6). Its toxic effect on renal tubule showed thickening of the glomerular basement membranes (GBMs), mild focal tubular necrosis, degenerative changes of mitochondria of convoluted tubule, interstitial nephritis and papillary necrosis with clinical manifestations as hematuria and proteinuria (7,8).

Carotenoids, flavonoids and phenolic acids possess antioxidant properties that reduce the damaging effects of oxidative stress, so they are considered as beneficial factors for preventing many human diseases among which cancer and cardiovascular pathologies (9). Chlorophyll is lipophilic antioxidants, 2 chlorophyll types were observed as chlorophyll a and chlorophyll b both present in plants, while chlorophyllin is a semi-synthetic mixture of sodium copper salts that is water soluble derived from chlorophyll (10). It can protect from diseases as atherosclerosis, osteoporosis, cataracts, and some types of cancer, neurodegenerative diseases,
mutagenesis, and oxidative stress by different mechanisms (11). Chlorophyllin, had high antioxidant activities compared to that was in natural chlorophylls, its antioxidant activity not only due to donate hydrogen abilities but also protecting linoleic acid against oxidations and prevented from hydroperoxides decomposition, in addition to activation of the endogenous xenobiotic detoxification systems (12). Vitamin D is included in different intracellular genomic activities, biochemical and enzymatic reactions. It has an essential role in overcoming inflammation, reduction of oxidative stress and controlling the aging process (14). Vitamin D has an antioxidant effect; as it regulates ROS levels, prevents lipid peroxidation in the cell membrane (15) and affect vitamin D axis and gene polymorphisms through vitamin D receptor (VDR) (16).

MATERIALS AND METHODS:

**Animals**:
32 Males (adult albino rats) which weights were 200-250 and purchased from animal house of Cairo University. Animals housed in cages in room temperature (22-25°C) in Pharmacology department lab, Faculty of Medicine, Fayoum University. With alternating light-dark cycle and free accessing to food /water. Ethical clearance get from Ethics Committee of the Faculty of Medicine, Fayoum University, and international ethics and regulations for animal research in laboratory applications.

**Chemicals**:
Diclofenac sodium ampoules for intramuscular (IM) injection was purchased from Pharmco (pharmaceutical company)
Vitamin D3 oral drops was purchased from Medical Union Pharmaceutical MUP (pharmaceutical company)
Chlorophyllin was purchased from Sigma-Aldrich (St. Louis, MO, USA)

**Experimental design**:
Rats divided by random way to 4 groups (each=8 rats)

**Group 1**: Control normal group: given normal saline for 4 weeks.

**Group 2**: Control analgesic group given IM injection of diclofenac Na 4 mg/kg [17] for 15 days for induction of analgesic nephropathy.

**Group 3**: Vitamin D3 group: given oral vit. D3 daily at 1000 IU/kg [18] /15 days before diclofenac Na injection and continued for another 15 days.

**Group 4**: Chlorophyllin group: given intraperitoneal (IP) injection of chlorophyllin daily at a dose of 30 mg/kg [19] for 15 days before diclofenac Na injection and continued for another 15 days.

**At the end of the experiment**:
After 24h of the final treatment, rats were anesthetized using injection of thiopental sodium 50 mg/ kg subcutaneously. Rats blood collected by retro-orbital puncture and centrifuging at 1000 rpm/10 minutes and serum was collected and stored at -70°C. All animals were sacrificed. Kidneys collected through making vertical midline abdominal incision. Post removed adherent connective tissues, one kidney from every rat stored on -20°C for PCR and contralateral kidney fixed in 10% formaldehyde for histopathological examination.

**Assessment of urea and creatinine**:
Serum urea and creatinine were determined by colorimetric method utilizing Kits produced by BioSystem SA Costa Brava, Barcelona, Spain, according to the manufacturer’s instructions.

**Assessment NADPH oxidase, Superoxide dismutase and IL-2**:

NADPH oxidase, Superoxide dismutase and IL-2 levels were detected in serum utilizing ELISA kits (DRG International Inc., Springfield, NJ, USA).

**Assessment of MDA and TNFα**:
MDA and TNFα levels were detected in kidney tissues utilizing ELISA kits (Calbiotech, Austin, USA).

All protocols followed the manufacturer’s instructions.

**DNA extraction and reverse transcription**: 
RNA isolated by guanidium isethionate utilizing Qiagen tissue extraction kit (Qiagen, CA, USA). Isolated RNA either purity or concentrations established by the NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA) and kept at -80°C. Reverse transcription of RNA was carried out utilizing QuantiTect reverse transcription kit (Qiagen) as described in manufacturer’s protocol.

Quantitative assessment of Caspase-3 and Nrf2 gene expression by real-time PCR. The expression of Caspase-3 and Nrf2 genes was estimated by RT-thermal cycler (M Research Inc, Waterton, USA) as explained in manufacturer’s protocol. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was employed as an internal control for data normalization. Primers utilized for PCR were displayed in Table 1.

<table>
<thead>
<tr>
<th>Table 1: Primer sequences for PCR.</th>
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</thead>
<tbody>
<tr>
<td><strong>Caspase-3</strong> sense: 5' TTCAGGCCCAAAAGAGCAGTTG-3'</td>
</tr>
<tr>
<td>antisense: 5'-GGGCTCAACATCTGCTTCAA-3'</td>
</tr>
<tr>
<td><strong>Nrf2</strong> sense: 5'-CCGTCGTAAGTTAAGGGCGACTTC-3'</td>
</tr>
<tr>
<td>antisense: 5'-GAGCTCAGGGACCCTAGTCCG-3'</td>
</tr>
<tr>
<td><strong>G3PDH</strong> sense: 5'-CCCCGCTGTGCTGCTGTCACC-3'</td>
</tr>
<tr>
<td>antisense: 5'-GTTCACCGAGAATGCGCAAC-3'</td>
</tr>
<tr>
<td><strong>RT-PCR</strong> was done in 25-ml reaction volume comprising 2X SYBR Green PCR Master Mix, 900 nM of each primer and 2ml of cDNA and involving 95°C step /10 minutes, followed by 40 cycles of 95°C/15 seconds, 60°C / 1 minute, and 72°C for 1 minute. Relatedly expressions of intended genes mRNA were calculated utilizing the 2-ΔΔCt method.</td>
</tr>
</tbody>
</table>

**Histopathology**: 
Kidney removed from rats and fixed in 10% formaldehyde solution. Tissue sections (4μm) mounted on glass slides and stained with hematoxylin-eosin to evaluate kidney structures and periodic acid-Schiff (PAS) for observation of basa lamina and brush border. Immunohistochemical staining was carried out using Ki-67 as a marker of cell proliferation [20] (Invitrogen). Thermo Fisher Scientific, USA) Rabbit Polyclonal Antibody (Catalog # PA5-16446), Immunoreactivity for Ki-67 was visualized as dark brown nucleus, the positive control was human tonsil tissue and HSP-70 [21] (Invitrogen. Thermo Fisher Scientific, USA) Mouse monoclonal antibody (Catalog # MAB3-008) where its immunoreactivity was cytoplasmic, positive control was human tonsil tissue. Additional slides of kidney specimens were treated with buffer solutions. Quantitative Morphometric Study:
The measurements done by using “Top view” image analyzer computer system (China). The mean area percent and optical density of both of PAS reaction and HSP-70 immunohistochemical staining; using an objective lens of magnification 40. The mean of Ki-67 immunopositive cells; interactive counting of immunopositive cells done by using lens of magnification 10. Digitalized images were captured from 10 randomly chosen non-overlapping fields from each section. |
Statistical Methods:
Statistical analysis was performed using the arithmetic mean, standard deviation (SD) after checking of normality of distribution, analysis of variance (one way ANOVA) and comparison between each two groups using post Hoc Tukey test, using the statistical SPSS software for Windows, Version 18 (USA). Significance was P < 0.05 [22].

RESULTS
Assessment of serum kidney function and oxidative parameters between different groups:
Significant increase in serum urea, creatinine, NADPH Oxidase and IL-2 levels in diclofenac group (56.33 ± 1.86, 1.41 ± 0.09, 268.17 ± 4.96 and 89.50 ± 1.87), respectively, when compared to control group (24.0 ± 0.89, 0.57 ± 0.10, 94.83 ± 0.75 and 35.51 ± 2.07; p < 0.05), in the same trend, this increase was significantly ameliorated by vitamin D3 (41.50 ± 5.54, 0.97 ± 0.12, 128.51 ± 9.04 and 52.17 ± 4.17) and chlorophyllin (44.83 ± 1.16, 1.12 ± 0.16, 179.01 ± 6.66 and 63.93 ± 2.48), while serum levels of SOD decrease in Diclofenac group (25.67 ± 1.21) when compared to control group (63.67 ± 3.50; p < 0.05) and this decrease improved by vitamin D3 (44.67 ± 3.20; p < 0.05) and chlorophyllin (42.01 ± 1.55; p < 0.05) as shown in table (1).

Assessment of tissue inflammatory markers between different groups.
Significant increase in MDA tissue level’s and TNFα in Diclofenac group (118.67 ± 4.03 and 147.01 ± 2.89), respectively, when compared to control group (55.01 ± 2.97 and 73.33 ± 3.27,), and this increase improved by vitamin D3 (74.83 ± 2.4 and 96.16 ± 2.78; p < 0.05) and chlorophyllin (82.67 ± 3.93 and 108.83 ± 8.26; p < 0.05) as shown in table (2).

Assessment of tissue Caspase-3 and Nrf2 gene expression between different groups.
Significant increase in gene expression of Caspase-3 in Diclofenac group (5.50 ± 0.31) when compared with control group (1.77 ± 0.19; p < 0.05), and this increase significantly ameliorated by vitamin D3 (2.56 ± 0.49) and chlorophyllin (2.57 ± 0.13), while gene expression of Nrf2 decreased in Diclofenac group (0.82 ± 0.02) when compared with control (1.45 ± 0.03; p < 0.05) and those decrease significantly improved by vitamin D3 (1.25 ± 0.03; p < 0.05) and chlorophyllin (1.11 ± 0.03; p < 0.05) as shown in table (3).

Histopathological results:
Hematoxylin & Eosin (H&E):
Examination of H&E-stained sections showed that control group (Fig. 1A) revealed the normal histological architecture of renal corpuscles, formed of Bowman’s capsules lined with simple squamous epithelia, glomeruli and normal capsular space. Proximal and distal tubules had normal epithelial lining. Diclofenac sodium treated group (Figs. 1B&C) showed retraction of glomerular tufts with its concomitant capsular space widening and vacuolization of the glomerular visceral epithelial cells. Proximal and distal tubules exhibited marked distorted structure. Others had epithelial vacuolations, nuclear pyknosis in addition to epithelial cell detachment that could be detected in some tubules. Some tubules had cystic dilatation others had intraluminal eosinophilic debris. Extravasations of erythrocytes in-between glomerular capillaries and tubules as well as inflammatory infiltration was also observed. Vitamin D3 treated group (Fig. 1D) appeared with normal histological morphology of the glomeruli and apparent normal tubules while capsular space widening could be detected. Chlorophyllin treated group (Fig. 1E) exhibited capsular widening and apparent normal tubules except for few dilated tubules. Epithelial lining cells in few tubules had vacuolations, pyknotic nuclei and few detached epithelial cells. Extravasated erythrocytes in-between glomerular capillaries and tubules, as well as inflammatory infiltration could be observed.

Periodic acid Schiff (PAS)
PAS-stained sections examination revealed that control group (Fig 2A) showed strong positive PAS reaction in Bowman capsule and renal tubules basement membranes as well as in the intact luminal brush border in majority of renal tubules. Absent reaction was noticed in luminal borders of few tubules while Diclofenac sodium treated group (Fig 2B) showed faint PAS reaction in basement membranes of Bowman capsule and renal tubules. Absence of PAS reaction in luminal borders of many tubules with faint reaction in of bush border of few tubules was an obvious finding. Both of Vitamin D3 (Fig 2C) and Chlorophyllin (Fig 2D) treated groups exhibited moderate positive PAS reaction in basement membrane of Bowman capsule, along the intact brush border and regular basement membrane of renal tubules whereas there was no reaction in luminal borders of few tubules.

HSP-70 immunostaining:
HSP-70 immunostained sections examination revealed that control group (Fig. 3A) showed strong immunoreaction in distal tubule epithelium and moderate cytoplasmic immunoreaction for HSP-70 in proximal convoluted tubules. Meanwhile Diclofenac sodium treated group (fig. 3B) exhibited faint immunoreaction in both of proximal and distal tubules. Vitamin D3 treated group (Fig3C) expressed moderate reaction in both of proximal and distal convoluted tubules. Chlorophyllin treated group (Fig 3D) appeared with strong immunoreaction in distal tubules and moderate immunoreaction in proximal tubules. The obvious finding was that negative reaction for HSP-70 immunohistochemical staining was observed in all groups.

Ki-67 immunostaining:
Ki-67 immunostained sections of examination revealed that control group (Fig. 4A) exhibited nuclear immunoreaction for Ki-67 mainly in tubular epithelial cells compared to the few epithelial lining cells of renal tubules that expressed Ki-67 in Diclofenac sodium treated group (Fig 4B). Whereas in vitamin D3 (Fig. 4C) and Chlorophyllin (Fig 4D) treated groups, there was nuclear immunoreactivity for Ki-67 in numerous glomerular and tubular epithelial cells.

Morphometric results: (Table 4)
Optical density and mean area % of PAS reaction:
Significant decrease in Optical density and mean area % of PAS reaction in diclofenac sodium group as comparing to other experimental groups. Significant difference was observed between both of vitamin D3 and chlorophyllin as compared with the control group. Regarding the optical density of PAS reaction there was non-significant difference between vitamin D3 and chlorophyllin groups whereas the area % of PAS reaction showed significant increase in vitamin D3 group as compared with chlorophyllin group.

Optical density and mean area % of HSP-70 immunoreactivity:
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Significant decrease in optical density and mean area % of HSP-70 immunoreaction in diclofenac sodium group as compared with the other groups. Meanwhile, significant difference was observed between both of vitamin D3 and chlorophyllin groups as compared with control group. No significant difference was detected between vitamin D3 and chlorophyllin groups.

Mean number of Ki-67 immunoreactive cells:

Figure 1: A photomicrograph of H&E-stained sections of the renal cortex from all experimental groups: Control group (A), showing normal histological architecture of renal corpuscles, formed of Bowman’s capsules lined with simple squamous epithelia (arrows), glomeruli (G) and normal capsular space (curved arrows). Proximal (PT) and distal tubules (DT) have normal epithelial lining; Diclofenac sodium treated group (B&C) showing retraction of glomerular tufts (G) with its concomitant capsular space widening (curved arrows) and vacuolization of the glomerular visceral epithelial cells (notch ended arrow). Proximal (PT) and distal (DT) tubules are exhibiting marked distorted structure (encircled by white lines). Others have epithelial vacuolations (V), nuclear pyknosis (arrowheads) and epithelial cell detachment (hollow arrows). Some tubules have cystic dilation (stars) others have intraluminal eosinophilic debris (dotted arrows). Extravasation of erythrocytes (right angled arrows) in-between glomerular capillaries and tubules, as well as inflammatory infiltration (encircled by black line) are obvious findings. Vitamin D3 treated group (D), showing normal histological morphology of the glomeruli (G), and apparent normal tubules (PT & DT) dilated capsular space (curved arrow) could be detected. Chlorophyllin treated group (E) showing capsular widening (curved arrows) and apparent normal tubules (PT &DT) except for few dilated tubules (star). Epithelial lining cells in few tubules have vacuolations (V), pyknotic nuclei (arrowheads) and detached epithelial cells (hollow arrows). Extravasated erythrocytes (right angled arrows) in-between glomerular capillaries and tubules, as well as inflammatory infiltration (encircled by black line) could be observed. (H&E stain, x400).
**Figure 2**: A photomicrograph of PAS-stained sections of the renal cortex from all experimental groups: Control group (A), showing strong positive PAS reaction in capsular basement membrane (dotted arrow), along the intact luminal brush border (arrows) and basement membrane (curved arrows) in majority of renal tubules. Absent reaction is noticed in luminal borders of few tubules (hollow arrow). Diclofenac sodium treated group (B) showing faint PAS reaction in basement membranes of Bowman capsule (dotted arrow) and renal tubules (curved arrow). Absence of PAS reaction in luminal borders of many tubules (hollow arrow) with faint reaction in of brush border of few tubules is an obvious finding. Vitamin D3 treated group (C) exhibiting moderate PAS reaction in renal tubules brush border (arrow) and prominent basement membrane of Bowman capsule (dotted arrow) and tubules (curved arrow) whereas there is no reaction in luminal borders of few tubules (arrow arrow). Chlorophyllin treated group (D) shows moderate positive PAS reaction in basement membrane of Bowman capsule (dotted arrow), along the intact brush border (arrows) and regular basement membrane (curved arrows) in majority of tubules. Few renal tubules exhibiting no luminal brush border PAS stain reaction (hollow arrow) (PAS stain, x400).

**Figure 3**: A photomicrograph of HSP-70 immunostained sections of the renal cortex from all experimental groups: Control group (A), showing negative immunoreaction in glomeruli (hollow arrows), moderate cytoplasmic immunoreaction for HSP-70 in proximal convoluted tubules (arrow) and strong immunoreaction in distal tubular epithelium (curved arrow).

Diclofenac sodium treated group (B) showing negative immunoreaction in glomeruli (hollow arrow) and faint immunoreaction in both of proximal (arrow) and distal (curved arrow) tubules. Vitamin D3 treated group (C) has negative immunoreaction in glomeruli (hollow arrow) and moderate reaction in both of proximal (arrow) and distal (curved arrow) convoluted tubules. Chlorophyllin treated group (D) appear with negative reaction in glomeruli (hollow arrows), moderate immunoreaction in proximal tubules (arrow) and strong immunoreaction in distal tubules (hollow arrows). (HSP-70 immunostain, x200).
Figure 4: A photomicrograph of Ki-67 immunostained sections of the renal cortex from all experimental groups: Control group (A), exhibiting nuclear immunoreaction for Ki-67 mainly in tubular epithelial cells. Diclofenac sodium treated group (B) shows nuclear reaction in few epithelial cells that line renal tubules. Vitamin D3 treated group (C), revealing nuclear immunoreactivity in numerous glomerular and tubular epithelial cells. Chlorophyllin treated group (D) showing many lining cells of both glomeruli and renal tubules that express nuclear immunostain for Ki-67. (Ki-67 immunostain, x200).

Table 1. Assessment of Serum parameters among different groups.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diclofenac</th>
<th>Diclofenac + Vitamin D3</th>
<th>Diclofenac + chlorophyll</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea (mg/dl)</td>
<td>24.0 ± 0.89</td>
<td>65.33 ± 1.86</td>
<td>41.50 ± 5.54</td>
<td>44.83 ± 1.16</td>
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<tr>
<td>Creatinine (mg/dl)</td>
<td>0.57 ± 0.10</td>
<td>1.41 ± 0.09</td>
<td>0.97 ± 0.12</td>
<td>1.12 ± 0.16</td>
</tr>
<tr>
<td>NADPH Oxidase (pg/ml)</td>
<td>94.83 ± 0.75</td>
<td>268.17 ± 4.96</td>
<td>128.51 ± 9.04</td>
<td>179.01 ± 6.66</td>
</tr>
<tr>
<td>SOD (pg/ml)</td>
<td>63.67 ± 3.50</td>
<td>25.67 ± 1.21</td>
<td>44.67 ± 3.20</td>
<td>42.01 ± 1.55</td>
</tr>
<tr>
<td>IL-2 (pg/ml)</td>
<td>35.51 ± 2.07</td>
<td>89.50 ± 1.87</td>
<td>52.17 ± 4.17</td>
<td>63.83 ± 2.48</td>
</tr>
</tbody>
</table>

Significance at P-value < 0.05
a: statistical significance compared to Control.
b: statistical significance compared to Diclofenac.
c: statistical significance compared to Vit D3.

Table 2. Assessment of tissue parameters among different groups.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diclofenac</th>
<th>Diclofenac + Vitamin D3</th>
<th>Diclofenac + chlorophyll</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol/g)</td>
<td>55.01 ± 2.97</td>
<td>118.67 ± 4.03</td>
<td>74.83 ± 2.4 a,b</td>
<td>82.67 ± 3.93 a,b</td>
</tr>
<tr>
<td>TNFα (pg/mg ptn)</td>
<td>73.33 ± 3.27</td>
<td>147.01 ± 2.89 b</td>
<td>96.16 ± 2.78 ab</td>
<td>108.83 ± 8.26 abc</td>
</tr>
</tbody>
</table>

Significance at P-value < 0.05
a: statistical significance compared to Control.
b: statistical significance compared to Diclofenac.
c: statistical significance compared to Vit D3.

Table 3. Results of Caspase-3 and Nrf2 gene expression among different groups.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diclofenac</th>
<th>Diclofenac + Vitamin D3</th>
<th>Diclofenac + chlorophyll</th>
</tr>
</thead>
</table>

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<table>
<thead>
<tr>
<th>Caspase-3 gene Expression</th>
<th>Control</th>
<th>Didclofenac sodium</th>
<th>Vitamin D3</th>
<th>Chlorophyll D3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.77 ± 0.19</td>
<td>5.50 ± 0.31a</td>
<td>2.56 ± 0.49a,b</td>
<td>2.57 ± 0.13a,b</td>
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<tr>
<td>Nrf2 gene Expression</td>
<td>1.45 ± 0.05</td>
<td>0.82 ± 0.02a</td>
<td>1.25 ± 0.03b</td>
<td>1.11 ± 0.03b</td>
</tr>
</tbody>
</table>

Significance at P-value < 0.05
a: statistical significance compared to Control.
b: statistical significance compared to Didclofenac.
c: statistical significance compared to Vitamin D3.

Table 4: Mean ± SD of optical density and area % of PAS reaction and HSP-70 immunoreaction, and mean number of Ki67 immunoreactive cells in renal cortex of all experimental groups:

<table>
<thead>
<tr>
<th>PAS reaction optical density</th>
<th>Control</th>
<th>Didclofenac sodium</th>
<th>Vitamin D3</th>
<th>Chlorophyll D3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.89±0.01</td>
<td>0.86±0.02a,b</td>
<td>0.85±0.01b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAS reaction area %</td>
<td>12.41±0.5</td>
<td>11.46±0.48b</td>
<td>8.21±0.47b,c</td>
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<tr>
<td>HSP-70 optical density</td>
<td>0.89±0.01</td>
<td>0.72±0.02a,b</td>
<td>0.85±0.01b,c</td>
<td></td>
</tr>
<tr>
<td>HSP-70 area %</td>
<td>6.57±0.45</td>
<td>7±0.50b</td>
<td>5.98±0.2a,b,c</td>
<td></td>
</tr>
<tr>
<td>Ki-67 mean number</td>
<td>99.3±5</td>
<td>24.38±1.41a</td>
<td>102±3.59b,c</td>
<td>74±3.2a,b,c</td>
</tr>
</tbody>
</table>

Significance at P-value < 0.05
a: Significant compared to control group.
b: Significant compared to Didclofenac sodium group.
c: Significant compared to Vitamin D3 group.

DISCUSSION

Diclofenac, a non-steroidal anti-inflammatory drug, is commonly used for its analgesic, anti-inflammatory, and anti-pyretic activities (23). Despite overspread using of diclofenac, renal injuries observed due to its administrations (24-26). In the present study, diclofenac treatment significantly elevated plasma creatinine and urea level compared with control group, indicating compromised renal function. which in accordance with Prince, (2018) (26). Diclofenac treatment led to ROS generation (reactive oxygen species), and increased oxidative stress (27). Therefore, antioxidant could attenuate ROS-mediated cellular damages. So, it can be as a therapeutic approach to prevent diclofenac cytotoxic effect (28).

NADPH oxidases, along with the mitochondria, are the major sources of oxidative stress in the kidney tissue (29), and tissue MDA is one of the molecules used as indicator of lipid peroxidation to estimate oxidative stress (30). From our results we found that, diclofenac treatment resulted in significant increase in NADPH Oxidase and renal MDA levels as compared to control group and antioxidant defenses reduced post diclofenac administrations and reflected by decreasing in SOD (superoxide dismutase) level. These are considered as reductions in body antioxidant and decrease in body defense mechanisms. Which in agree with observes of Giridharan et al. (31) who found that, significant deterioration in renal function and oxidative markers post diclofenac injections.

Chlorophyllin, a water-soluble sodium copper salt derive from chlorophyll, is a compound which used as food dye and wound-healing accelerant (32).

In the present study, treatment of rats with chlorophyllin and vitamin D3 resulted in significantly decreasing in creatinine plasma levels of and urea indicating improvement in kidney functions. Moreover, they reduced oxidative stress and lipid peroxidation in renal tissues indicated by significant decrease in renal MDA level and serum NADPH Oxidase. In addition, there was elevation in renal antioxidant enzymes (SOD) level due to chlorophyllin or Vitamin D3 administration. Pervious investigations were support that, chlorophyll and its derivatives have antioxidant properties. (33). The role played by vitamin D in ameliorating the oxidative stress also has been investigated by many studies. Kim et al. found that, 1, 25(OH)2 D3 induced the up-regulation of antioxidant expression in endothelial cells (34). Vitamin D signaling pathway also has been proved to play a vital role in cells protection against elevated mitochondrial respiration and over production of reactive Oxygen species (ROS) that lead to cellular and DNA damage (35). The nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor, can potently activates expressions of multiple antioxidant and detoxifying genes of ROS in chronic renal failure and renal inflammation. These effects are due to binding of Nrf2 to antioxidant-response elements (ARE) in the promoter region of a number of genes encoding antioxidant and phase 2 metabolizing enzymes (36, 37). Furthermore, Nrf2 is negatively regulated by Kelch ECH associating protein 1 (Keap1). Keap1, a repressor protein binds to Nrf2 and promotes its degradation. The Keap1-Nrf2 pathway is the major regulator of cytoprotective responses to endogenous and exogenous stresses caused by ROS (38). In the present study, gene expression of Nrf2 was significantly decreased in diclofenac group when compared to control group, while in CHL and vitamin D treated groups a significant increase was observed. Many studies tried to explain the mechanism by which chlorophyllin exerts its antioxidant effect; Fahey et al. (39) demonstrated that chlorophyllin has the ability to induce mammalian phase 2 proteins that protect cells against oxidants and electrophiles via activation of the ARE/Nrf2 pathway and this, in part, explains the widely accepted protective effects of vegetable consumption against cancer. The
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underlying mechanism of the diminishing effect of VIT.D on the oxidative stress was the inhibition of NADPH oxidase expression and enhancement in cytosolic SOD enzyme (40). Decreased level of oxidative markers including MDA, nitric oxide and protein carbonyl groups in serum and increased level of antioxidant defenses containing glutathione (GSH) peroxidase, catalase and SOD activity after Vitamin D treatment in hemodialysis patient have been noticed (41). Lisse, (53) reported that concomitant decreasing in catalase and SOD1 mRNA, and increasing in SOD2 mRNA post one day of 10 nM 1,25(OH)2D3 treatment and ~8-fold increase in SOD2 mRNA was apparent after 48 ours. Oxidative stress and inflammation are closely related process and inflammation is oxidative stress consequences (43). Over producing the free radicals initiate producing nuclear factor-kappa B and intracellular signaling cascade (44). TNF-α exerts pro-inflammatory effects like endothelial apoptosis, induction of oxidative stress, upregulation of adhesion molecules and chemokines (45). It is reported that strongest prostaglandin synthase inhibitors as diclofenac causes increase levels of TNF-α. In our study, diclofenac treatment triggered a significant increase in the level of pro-inflammatory cytokines IL-2 and TNF-α in the kidney of treated rats; indicating formation of oxidative stress and inflammation. This is in accordance with Alabi and Akomolafe (28).

The present study showed that chlorophyllin and Vit. D3 produced significant decrease in serum IL-2 level and in renal TNF-α level. This is in agreement with Subramoniam et al. (47) who demonstrated that chlorophyllin showed potent anti-inflammatory activity against carrageenan-induced paw edema in mice and formalin-induced paw edema in rats. Chlorophyllin also showed remarkable inhibition of TNF-α gene expression in a concentration-dependent manner. They postulated that the anti-inflammatory mechanism of chlorophyllin is likely to be due to their influence on the expression of the pro-inflammatory cytokine, TNF-α. Moreover, Nrf2 is anti-inflammatory, as evidenced by several observations; Nrf2 knockout mice have a tendency to develop age-dependent autoimmune and inflammatory lesions in multiple tissues (48). Inflammation is commonly observed in chemically induced pathology with Nrf2 deficiency (49, 50) and inhibition of inflammation by Nrf2 is associated with inhibition of the NF-kB pathway and inhibition of pro-inflammatory cytokine production (51). Thus, our study indicated that the anti-inflammatory property of chlorophyllin could be related to its ability to down-regulate the production of IL-2 and TNF-α as well as up-regulation of Nrf2 gene. Vitamin D exerts anti-inflammatory effect through repression of transcription of genes encoding IL2 (52). Furthermore, Aljjack et al. (53) suggested that Vit. D deficiency may contribute to systemic inflammation, because vitamin D inhibited Th-1 lymphocytes to produce some inflammatory markers like interferon-gamma, IL-2 and IL-5. Vit. D prevented the upregulated inflammatory cytokines such as TNF-alpha, INF-gamma, IL-1beta, and the adhesion molecules such as monocyte chemoattractant protein-1. (54). Several mechanisms by which Vitamin D3 exerting anti-inflammatory effects included regulating the interaction between immune cells to regulate the levels of cytokines via caspase-3 signaling pathway, up-regulating MKP5, and inhibiting the prostaglandins pathway and immune cells (55).

In the present study, caspase-3 activation, which is mandatory for apoptosis to occur, was confirmed by evaluation of caspase-3 gene expression by RT-PCR. Caspase-3 is the main downstream effector caspase that cleaves the majority of the cellular substrates in apoptotic cells (56). In the present study, there was a statistically significant increase in the level of caspase-3 expression in diclofenac treated rats compared to control rats. This agreed with other work that found that diclofenac activates caspase-3 and attributed that to oxidative stress with release of reactive oxygen species (ROS) subsequently (57). Apoptotic stimuli cause release of cytochrome-c from mitochondria that encourage a series of reactions, causing activation of caspase, which leads to consequent cell death (58). Accumulating suggestions propose that apoptosis plays a crucial role in numerous mechanisms of renal injury (59).

The current study revealed that chlorophyllin and Vitamin D3 significantly decreased gene expression of caspase 3 that was induced by diclofenac treatment indicating inhibition of apoptosis. This is in agreement with the study of Patar et al. (60) that showed that chlorophyllin down regulated caspase 3 and caspase 9, whereas upregulated Bcl-2 protein demonstrating a few apoptotic cells in liver of diabetic mice. They postulated that chlorophyllin treatment can exert hepatoprotective effect via modulating hyperglycemia-induced oxidative stress and apoptosis in STZ-administered diabetic mice. Notably, the modulation of apoptosis by chlorophyllin is evident from several studies (61, 62). Moreover, in the present study, upregulation of Nrf2 expression by chlorophyllin provided more evidence to its antiapoptotic effect in renal cells. Nrf2 was shown to induce expression of antiapoptotic molecules such as Bcl-2 enhancing cell survival (63). In another study by Yang et al. (64) renal activation of Nrf2 expression resulted in the prevention of apoptosis in renal cells and reduced levels of activated caspase-3 and caspase-9 in renal tissues. In our study, chlorophyllin showed a cell survival mode of actions against apoptosis induced by diclofenac treatment as evident by down-regulation of caspase 3 whereas upregulation of Nrf2 expressions suggesting that one of the mechanisms involved in the renoprotective effects of chlorophyllin was via activation of the Nrf2-mediated antioxidant pathway in the kidney. Vitamin D treated group observed a significant decrease in caspase3. This finding is in agreement with Suh et al. (65) who reported that increased cleaved form of caspase-3 in Gentamicin group detected by immunoblotting technique was reversed by paricalcitol treatment. Abd El-Hafiz et al. (66) also reported a significant decrease in the caspase-3 expression on administration of calcitriol in diabetic rats which ameliorates the diabetic nephropathy.

This study revealed marked histopathological changes in diclofenac sodium treated group that was manifested by both of glomerular and tubular damage. There was an association of these histological alterations and the significant increases in NADPH, IL-2, MDA and TNF-α along with the significant decrease of SOD and Nrf2 levels. This may clarify the possible pro-inflammatory and oxidative capability of Diclofenac in inducing renal damage via causing mitochondrial injury and production of reactive oxygen metabolites. The toxic effects of diclofenac could be also inhibited by inhibiting Nrf2, which attenuated Nrf2-mediated antioxidant action (67) that would lead to renal arteries constriction (68). The obvious apoptosis that was detected in the tubular epithelium could be
explained by both DNA damage and oxidative stress (69). Shedding of degenerated cells of renal tubules leads to accumulation of intraluminal eosinophilic debris (70). Vacuolations in tubular lining cells that were detected in diclofenac group may be attributed to the induced cellular damage as revealed by Filippopulos and Vlassopulos, (71) who stated that the increased cell membrane permeability results in increased cellular diffusion and water accumulation. Inflammation and inflammatory mediator’s activation pathways are considered as consequences of oxidative stress (72) and this could explain the detected inflammatory infiltration in Diclofenac group. Similar findings were reported by El-Maddawy and El-Ashmawy, (73) who declared that diclofenac administration caused marked renal tissue inflammation.

The histopathological changes detected in this study were in accordance with those of Soha et al., (74), Owumi & Dim, (75) and Alabi & Akomolafe, (28) who revealed similar findings in renal cortex in the form of glomerular and tubular injury induced by Diclofenac administration. Treatment with Vitamin D revealed relative restored tubules and glomeruli with dilated Bowman’s space. Similar findings were detected by Dabak et al. (76) who declared that vitamin D had a satisfactory tubulointerstitial recovery in Adriamycin-induced nephrotoxicity in rats. The protection that was attained by vitamin D3 likely to be associated with antioxidant and anti-inflammatory effects of vitamin D. Gurel et al., (77) reported that vitamin D decreased nephropathy and apoptosis induced by doxorubicin and they explained this improvement by vitamin D antioxidant capacity. Moreover, vitamin D treatment significantly decreases inflammatory infiltration and apoptotic process in mesangiproliferative glomerulonephritis model (78) and prevented podocyte damage in experimental model of puromycin aminonucleoside nephrosis (79).

Chlorophyllin treated group offered some protection against the histopathological changes in the form of relative preserved glomeruli and tubules. Some extravasated erythrocytes in-between glomerular capillaries and tubules were observed with dilated Bowman’s space. Some tubules showed cystic dilatation with loss parts of lining epithelium. Similar findings were reported by Abdel Latif et.al. (80) on their administration of chlorophyll to mitigate the renal toxicological effects of aflatoxin.

Chlorophyllin ameliorating effect can be attributed to its antioxidant capacity for prevention of lipid peroxidation (81). Suparni et al. (33) have reported potential curative effect of chlorophyll obtained from Anagropogon leaves on biochemical, hematomalogical, and histological alterations induced by sodium nitrate exposure in rats. They declared that Chlorophyll has a remarkable antioxidant capability.

PAS reaction showed significant decrease (represented by area % and optical density) in diclofenac group as compared with the control and other treated groups. This decrease in PAS reaction is consistent with the same results detected by Mustafa et al. (82). This might be due to necrosis, and apoptosis that lead to loss of microvilli and the detachment of tubular cells from the basement membrane, leaving behind areas of denuded basement membranes (83). This process is of great importance for cellular repair following sublethal cellular injury (84).

Regarding HSP-70 immunoreaction, a significant increase (represented by area % and optical density) was detected in diclofenac group as compared with control and other treated groups. Down-regulation of HSP-70 could lead to kidney diseases due to the decrease of immunoregulatory, anti-inflammatory as well as chaperone function of HSP-70 (85, 86). An obvious immunoreaction of HSP-70 was detected in renal tissue of control group in this study; this finding is in accordance with that was found by Pedrycz & Siermontowski (87), they suggested that healthy control rats might be exposed to environmental stressors which couldn’t be removed totally as well as, it is essential to healthy cells to contain enough amounts of chaperones for protection of mature and newly formed cellular proteins.

Vitamin D3 has been documented to induce HSP-70 expression in rat kidney in a nontoxic manner (88), and this explains the marked increase of HSP-70 in vitamin D3 treated group. Xu et al., (2015) showed that that Vit D3 pretreatment down-regulated renal inflammatory response in acute kidney injury induced by lipopolysaccharide. Vitamin D receptor (VDR) binds 1, 25(OH)2 D3 to mediate vitamin D actions (89). Garcia et al. (90) suggested that HSP-70 as a cytoprotective agent is a key factor in the regulatory mechanism between vitamin D receptor and the primary component of the renin-angiotensin system. The significant increased expression of the cytoprotective HSP-70 in chlorophyll group revealed the protective efficacy of chlorophyll. This study showed an apparent increase in immunostaining of HSP-70 in distal convoluted tubules (DCT) more than proximal convoluted tubules (PCT). This discrepancy can be explained by the previous experimental data that revealed that PCT are greatly affected by diclofenac (28, 82). PCT cells were frequently affected by disturbed cellular metabolic pathways and these cells are greatly vulnerable to nephrotoxic agents (91).

Ki-67 immunostaining is a marker of cellular proliferation as ribosomal RNA transcription is associated with Ki-67. Diclofenac group in this study revealed significant decrease in Ki-67 expression as compared with the control and other treated groups. This decrease of cellular proliferation could be explained by the induced DNA damage by diclofenac exposure (69). The significant increase in cell proliferation as revealed by Ki-67 immunostaining in both vitamin D3 and Chlorophyllin groups denotes the potential protective effects of vitamin D as well as Chlorophyll against the adverse effects of diclofenac.

CONCLUSION

It can be stated that chlorophyllin or vitamin D3 treatment can exert renoprotective effect in diclofenac induced renal impairment in rats via modulating oxidative stress and apoptosis in addition to anti-inflammatory effect. Despite the protective effects of both vitamin D3 and Chlorophyllin, none of them succeeded in restoring the kidney to the normal as compared with the control group. It is worth mentioning that there was a significant difference between Vitamin D3 and Chlorophyllin treated groups as regard Ki 67 immunoeexpression and PAS reaction. This observation may make vitamin D3 superior on chlorophyllin. This might be due the used doses of different treatment. As in this study we have used a very high dose of vitamin D3, while chlorophyllin was used in low dose in comparison.

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