Evaluation of reno-protective effect of grape seed proanthocyanidin extract on Cyclosporine A-induced Nephrotoxicity by mitigating inflammatory response, oxidative stress and apoptosis in rats

Samy A. Hussein1, Yakout A. El-Senosi1, Tahia E.A. Esmael2, Aziza Amin1, Eman A.M. Sarhan1*

1 Department of Biochemistry, Faculty of Vet. Med., Benha University, Egypt.
2 Department of Nutrition and Clinical Nutrition, Faculty of Vet. Med., Benha University, Egypt.
3 Department of pathology, Faculty of Veterinary Medicine, Benha University, Egypt.

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ABSTRACT
Cyclosporine A (CsA) is a drug used as immunosuppressive agent in organ transplant and non-transplant medicine. The main secondary effect results from CsA treatment is nephrotoxicity. A protective effect of Grape seed proanthocyanidin extract (GSPE) against CsA-induced nephrotoxicity in rats was assessed. Thirty male rats were divided into three equal groups. Group I: (Normal control), received no drugs. Group II: (CsA treated), rats received oral dose of CsA (25 mg/kg b.wt/day) for 21 successive days. Group III: (GSPE protected+ CsA), rats received GSPE (200 mg/kg b.wt/day) orally 7 days before and during 21 days of CsA treatment. The obtained results showed a significant increase in serum urea and creatinine concentration in addition to L-MDA levels in kidney tissue while a marked decrease in renal catalase activity and GSH concentration in CsA treated rats. Moreover, a significant down-regulation in Bcl-2 and up-regulation of NF-κB, PAI-1, Caspase-3 and p53 gene expressions were observed in kidney tissues of CsA treated rats. Meanwhile, GSPE potentially improved renal function and oxidative alterations related to CsA near its normal ranges. Also, various histopathological alterations were detected in kidneys of CsA treated rats. Interestingly, histopathological findings supported that where GSPE markedly attenuated the harmful effects induced by CsA and protected kidney tissues. Our research could conclude that, GSPE has an ameliorating role as potent antioxidant, anti-inflammatory and anti-apoptotic agent via inhibition of inflammatory (NF-κB, PAI-1) and apoptotic (Caspase-3, p53) signaling pathway in modulation of CsA-induced nephrotoxicity.

1. INTRODUCTION

Cyclosporins are immunosuppressant drugs isolated from Tolypocladium inflatum (Cragg and Newman, 2013). Cyclosporine A nephrotoxicity is related to its chronic use in numerous autoimmune diseases and in organ transplantation especially kidney transplantation (Racisi et al., 2016). Oxidative stress has important role in Cyclosporine A nephrotoxicity. Cyclosporine A-induced nephrotoxicity results from the endoplasmic reticulum stress activation, the increased production of the mitochondrial reactive oxygen species (ROS) which modifies the redox balance enhancing lipid peroxidation ends with nephrotoxicity (Wu et al., 2018).

Procyanidins are the most common flavonoids present in grapes. Grape seed proanthocyanidin extract (GSPE) is rich in polyphenols which play an important role as a metabolic regulator and reactive oxidative species (ROS) scavenger (Gil-Cardoso et al., 2017). GSPE significantly protects against oxidative stress damage more effective than vitamins C, E and beta-carotene (Hassan et al., 2013). This study aims to evaluate the possible protective effect of grape seed proanthocyanidin extract (GSPE) against the nephrotoxic effect of Cyclosporine A long term administration in adult male rats through investigation of renal functions, oxidative stress and antioxidant biomarkers, pro-inflammatory mediators, apoptotic and anti-apoptotic gene expression markers in kidney tissues in addition to histopathological alterations of kidneys.

2. MATERIAL AND METHODS

2.1. Experimental animals:
Thirty-white male albino rats, 4-5 weeks old with average body weight 140-160 g were used in this study. Rats were fed on constant ration and fresh, clean drinking water was supplied ad-libitum. All rats were acclimatized for two weeks prior to the beginning of study. The experimental protocols were approved by the Animal Care and Use committee at Benha University and are in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.2. Chemicals and antioxidant agent:
2.2.1. Cyclosporine (CsA): CsA presents in the form of soft gelatin capsules containing 50 mg cyclosporine under traditional name (Sandimmune®, Neoral®) obtained from (Novartis Pharma AG, Basilea, Suiza). CsA was freshly dissolved in propylene glycol. Nephrotoxicity induced in

* Corresponding author: Eman A. M. Sarhan; E-mail: Flowerodour@gmail.com
rats after oral treatment with CsA (25 mg/kg b.wt/day) for 21 successive days according to Chia et al., (2012). 2.2.2. Grape Seed proanthocyanidin Extract (GSPE): GSPE was purchased from (Al Debeiky Pharma Company for Pharmaceutical industries, Al Obour, Cairo, Egypt). GSPE was dissolved in Dimethyl sulfoxide (DMSO) (100%) and diluted to the appropriate concentration by sterilized saline solution similar to previous study (El-Gawish et al., 2006).

2.3. Experimental design:
Rats were randomly divided into three groups (10 rats each) placed in individual cages and classified as follow:
2.3.1. Group I: (Normal control group): Rats fed only with ordinary diet without any treatment during the experimental period.
2.3.2. Group II: (Cyclosporine A nephrotoxic group): Rats orally received CsA (25 mg/kg b.wt/day) for 21 successive days.
2.3.3. Group III: (GSPE protected group): Rats orally received GSPE at a dose of (200 mg/kg b.wt/day) 7 days prior to CsA administration and concurrently during CsA treatment for 21 days later.

2.4. Sampling:
2.4.1. Blood samples
Blood sample was collected from retro-orbital plexus of eyes puncture. Blood was allowed to clot then centrifuged for 15 minutes at 3,000 rpm. Sera were separated in dry sterile tubes by automatic pipette, and then stored at -20 °C in a deep freezer until determination of urea and creatinine.
2.4.2. Tissue samples:
After blood collection rats were sacrificed by decapitation according to Animal Ethics Committees and abdomen was opened, then kidneys were collected.
2.4.3. For biochemical analysis:
Briefly, one gram of kidney tissues were cut and minced into small pieces, homogenized with a glass homogenizer in 9 volume of ice-cold 0.05 mM potassium phosphate buffer (pH7.4) to make 10% homogenates, then centrifuged for 15 minutes at 6000 r.p.m at 4°C. The supernatant used for determination of reduced glutathione (GSH), catalase (CAT), and L-malondialdehyde (L-MDA).
2.4.4. For molecular analysis (kidney):

### RESULTS

#### 3.1. Biochemical findings

Data were presented in table (2) that, serum urea and creatinine were significantly elevated in CsA rats when compared with normal control group. However, GSPE significantly reduced these concentrations comparing with CsA nephrotoxic group. The obtained results in table (3) showed a significant increase in L-MDA concentration and marked decrease in GSH concentration and CAT activity in kidney tissue of CsA treated rats as compared with normal control group. About 0.5 g of kidney tissue was put in Eppendorf tubes, kept immediately in liquid nitrogen and stored at -80°C till RNA extraction for determination of caspase-3, tumor suppressor protein p53, B cell lymphoma-2 (Bcl-2), nuclear factor kappa B (NF-κB) and plasminogen activator inhibitor-1 (PAI-1) gene expressions.

#### 2.4.5. For histopathological examination:
Small tissue specimens were collected from the kidneys of rats in all groups and then immediately fixed in 10% neutral buffered formalin solution then subjected to histopathological examination according to the technique described by Bancroft and Gamble, (2008).

#### 2.5. Analysis:
2.5.1. Biochemical analysis
Serum urea and creatinine were measured according to described methods by Kaplan and Kohn (1992) and Jaffe, (1986), respectively. Also, kidney tissue catalase activity, GSH and L-MDA concentrations were measured according to described methods by Weydert and Cullen (2010), Moron et al. (1979) and Lahouel et al. (2004), respectively.

2.5.2. Molecular analysis:
The mRNA expression contents of Caspase-3, p53, Bcl-2, NF-κB and PAI-1 were determined using real time quantitative polymerase chain reaction analysis (real-time qPCR) in rat kidney. Forward and reverse primers sequence for real time PCR is shown in table (1).

#### Table 1: Forward and reverse primers sequence for real time PCR.

| Gene      | Forward primer (5'------3') | Reverse primer (5'------3') |
|-----------|-----------------------------|-----------------------------|
| Caspase-3 | GCTATTGAGACAGACAGACTGCG     | CATGGGATCGTTCCTGGG          |
| p53       | ATGGCTTCCACCTGCGCTTC        | TGACACCAACTGACAGGCGG       |
| Bcl-2     | ATGGCTTCTGGGTGATGACTGAGTAC  | AGAGACAGGCAGGAATCAAAC         |
| NF-κB     | CCTACCTTTTCTGTAAGCTCAGAAA   | GGATACAGGCGCAATAGAGA         |
| PAI-1     | TTTTCCTACAGGCCATCATTCTCT    | GAAAGATCGTTCATAAACCATCCT    |
| B-actin   | AAGCTCCTCACCTCCCTCCAAGG     | AAAGCAATGCCTACTCCCTCC       |

#### 3.2. Statistical analysis:
Results were expressed as mean ± SE using SPSS (13.0 software, 2009). Data were analyzed using one-way ANOVA followed by Duncan’s test. Values were statistically significant at p < 0.05.

#### Table 2: Changes in biochemical parameters in diabetic rats treated with or without GSPE (n = 10 rats/group).

| Group                      | Serum urea (mg/dl) | Creatinine (mg/dl) | GSH (μmol/g) | L-MDA (μmol/g) | p-value |
|----------------------------|--------------------|--------------------|--------------|----------------|----------|
| Normal control             | 15.2 ± 2.3         | 8.5 ± 1.2          | 43.5 ± 2.7   | 0.6 ± 0.1      | 0.12     |
| CsA nephrotoxic            | 25.7 ± 3.2         | 12.0 ± 1.8         | 29.0 ± 1.5   | 1.2 ± 0.2      | < 0.05   |
| GSPE protected             | 18.2 ± 2.1         | 9.8 ± 1.4          | 39.0 ± 2.3   | 0.8 ± 0.1      | 0.01     |

#### Table 3: Changes in GSH and L-MDA concentrations in diabetic rats treated with or without GSPE (n = 10 rats/group).

| Group                      | Time | GSH (μmol/g) | L-MDA (μmol/g) | p-value |
|----------------------------|------|--------------|----------------|----------|
| Normal control             | 1    | 43.5 ± 2.7   | 0.6 ± 0.1      | 0.12     |
| CsA nephrotoxic            | 12   | 29.0 ± 1.5   | 1.2 ± 0.2      | < 0.05   |
| GSPE protected             | 12   | 39.0 ± 2.3   | 0.8 ± 0.1      | 0.01     |

#### Table 4: Changes in caspase-3, p53, Bcl-2, NF-κB and PAI-1 gene expressions in diabetic rats treated with or without GSPE (n = 10 rats/group).

| Gene      | Time | p-value |
|-----------|------|---------|
| Caspase-3 | 12   | < 0.05  |
| p53       | 12   | < 0.05  |
| Bcl-2     | 12   | < 0.05  |
| NF-κB     | 12   | < 0.05  |
| PAI-1     | 12   | < 0.05  |

#### Table 5: Changes in histopathological findings in diabetic rats treated with or without GSPE (n = 10 rats/group).

| Group                      | Time | Changes |
|----------------------------|------|---------|
| Normal control             | 12   | Normal  |
| CsA nephrotoxic            | 12   | Severe  |
| GSPE protected             | 12   | Mild    |

About 0.5 g of kidney tissue was put in Eppendorf tubes, kept immediately in liquid nitrogen and stored at -80°C till RNA extraction for determination of caspase-3, tumor suppressor protein p53, B cell lymphoma-2 (Bcl-2), nuclear factor kappa B (NF-κB) and plasminogen activator inhibitor-1 (PAI-1) gene expressions.

#### 2.4.5. For histopathological examination:
Small tissue specimens were collected from the kidneys of rats in all groups and then immediately fixed in 10% neutral buffered formalin solution then subjected to histopathological examination according to the technique described by Bancroft and Gamble, (2008).

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2.5.1. Biochemical analysis
Serum urea and creatinine were measured according to described methods by Kaplan and Kohn (1992) and Jaffe, (1986), respectively. Also, kidney tissue catalase activity, GSH and L-MDA concentrations were measured according to described methods by Weydert and Cullen (2010), Moron et al. (1979) and Lahouel et al. (2004), respectively.

2.5.2. Molecular analysis:
The mRNA expression contents of Caspase-3, p53, Bcl-2, NF-κB and PAI-1 were determined using real time quantitative polymerase chain reaction analysis (real-time qPCR) in rat kidney. Forward and reverse primers sequence for real time PCR is shown in table (1). β-actin was used as load control. Total RNA was isolated from the kidney using High Kit for isolation of pure RNA (Thermo Scientific, Fermentas, #K0731) RNA Extraction kit according to manufacturer’s instructions. With each cDNA sample was reverse transcribed using RevertAid TM First Strand CDNA synthesis kit (#EP0451, Thermo Scientific, Fermentas, USA). Then, real-time quantitative PCR amplification was performed on Faststart Universal SYBR Green Master (Roche, GER). The target gene was normalized with β-actin by the 2^-ΔΔCt method (Livak and Schmittgen, 2001).

2.6. Statistical analysis:
Results were expressed as mean ± SE using SPSS (13.0 software, 2009). Data were analyzed using one-way ANOVA followed by Duncan’s test. Values were statistically significant at p < 0.05.
kidney gene expressions in CsA treated rats when compared to normal control group. Meanwhile, GSPE administration before and during CsA treatment resulted in significant down-regulation in Caspase-3, p53 and up-regulation in Bcl-2 kidney gene expressions when compared with CsA nephrotoxic group.

Table 2 The Protective effect of GSPE on serum urea and creatinine concentrations in Cyclosporine A-induced nephrotoxicity in rats.

| Animal groups | Urea (mg/dl) | Creatinine (mg/dl) |
|---------------|--------------|-------------------|
| Group I: Normal control | 35.44 ± 0.13 | 0.92 ± 0.01 |
| Group II: CsA group | 59.07 ± 0.23 | 1.58 ± 0.06 |
| Group III: GSPE + CsA | 44.40 ± 0.31 | 1.36 ± 0.02 |

Data are presented as (Mean ± S.E). S.E = Standard error. Mean values with different superscript letters in the same column are significantly different at (P ≤ 0.05).

Table 3 The Protective effect of GSPE on kidney tissue L-MDA, GSH concentrations and CAT activity in Cyclosporine A-induced nephrotoxicity in rats.

| Animal groups | L-MDA (mmol/g. tissue) | GSH (mg/g. tissue) | CAT (U/g. tissue) |
|---------------|------------------------|-------------------|------------------|
| Group I: Normal control | 3.34 ± 0.09 | 34.75 ± 0.32 | 9.75 ± 0.15 |
| Group II: CsA group | 17.03 ± 0.35 | 17.77 ± 0.28 | 2.41 ± 0.15 |
| Group III: GSPE + CsA | 10.45 ± 0.22 | 23.08 ± 0.57 | 7.43 ± 0.22 |

Data are presented as (Mean ± S.E). S.E = Standard error. Mean values with different superscript letters in the same column are significantly different at (P ≤ 0.05).

Table 4 The Protective effect of GSPE on kidney tissue NF-kB and PAI-1 gene expression levels in Cyclosporine A-induced nephrotoxicity in rats.

| Animal groups | Nuclear factor kappa B (NF-kB) | Plasminogen activator inhibitor-1 (PAI-1) |
|---------------|-------------------------------|------------------------------------------|
|               | Fold change                   | Fold change                              |
| Group I: Normal control | Mean ± SEM                   | Mean ± SEM                               |
| Group II: CsA group | 1.00 ± 0.04                  | 0.07 ± 0.04                              |
| Group III: GSPE + CsA | 4.35 ± 0.21                  | 0.21 ± 0.04                              |

Data are presented as (Mean ± S.E). S.E = Standard error mean. Mean values with different superscript letters in the same column are significantly different at (P ≤ 0.05).

Table 5 The Protective effect of GSPE on kidney tissue apoptotic (Caspase-3, p53) and anti-apoptotic (Bcl-2) gene expression levels in Cyclosporine A-induced nephrotoxicity in rats.

| Animal groups | Caspase-3 | Tumor suppressor protein P53 | Bcl-2 |
|---------------|----------|------------------------------|-------|
|               | Fold change | Fold change | Fold change |
| Group I: Normal control | Mean ± SEM | Mean ± SEM | Mean ± SEM |
| Group II: CsA group | 0.07 ± 0.06 | 1.00 ± 0.06 | 0.06 ± 0.05 |
| Group III: GSPE + CsA | 6.2 ± 0.27 | 3.92 ± 0.15 | 0.15 ± 0.14 |

Data are presented as (Mean ± S.E). S.E = Standard error mean. Mean values with different superscript letters in the same column are significantly different at (P ≤ 0.05).

3.2. Histopathological examination:
Microscopical examination of kidney tissues obtained from normal control rats (Group I) revealed normal histological structure with normal glomeruli and proximal and renal tubules. The examined renal tissues obtained from CsA treated rats in (Group II) revealed various histopathological alterations including congestion of inter-tubular blood capillaries and renal blood vessels (Figure 1a) with perivascular mononuclear leukocytic cellular infiltrations mainly lymphocytes and macrophages in renal cortex with vacuolar degeneration in the wall of renal blood vessels (Figure 1b). Vasculature of the lining endothelial cells of the glomerular tuft as well as Hyper-cellularity of the glomerular tuft with adhesions between Bowman’s capsule and glomerular tufts were seen. (Figure 1c). Accidentally, necrosis, shrinkage and disintegration of the glomerular tuft were also observed (Figure 1c). The lining epithelium of some proximal and distal convoluted tubules in renal cortex exhibited extensive degenerative changes such as vacuolar and hydropic degeneration with necrosis of the lining epithelium of other renal tubules (Figure 1d,e). Meanwhile, microscopical examination of the kidney tissues obtained from the protected group with GSPE (Group III), revealed moderate improvement in the renal tissue histology as mild congestion of the inter-tubular blood capillaries and renal blood vessels with proliferation and hyper-cellularity of the endothelial cell lining of the glomerular tuft with adhesions between Bowman’s capsule and glomerular tufts and absence of Bowman’s spaces were detected (Figure 1e). Multifocally, the lining epithelium of some proximal and distal convoluted tubules in renal cortex exhibited mild degenerative changes such as cloudy swelling (Figure 1f).

4. DISCUSSION
Nephrotoxicity could be the major problem in transplant medicine as a side effect caused by Cyclosporine A (CsA) when used as an immunosuppressive drug. Oxidative stress has a crucial role in nephrotoxicity induced by Cyclosporine A (Raeisi et al., 2016). Apoptosis increased oxidative stress, and mitochondrial dysfunction were reported in CsA nephrotoxicity (Hausenloy et al., 2012). Renal failure was the end stage resulted from nephrotoxicity associated with long term administration of Cyclosporine A (Caires et al., 2018). Cyclosporine A treated rats revealed a significant increase in serum concentration of urea and creatinine. Abdel-Wahab, (2015) demonstrated that CsA administration for 21 days impaired the proper kidney function and induced the renal oxidant/antioxidant homeostasis disturbance. Urea and creatinine levels were increased, while glomerular filtration rate (GFR) was decreased on chronic CsA administration. This was related to reactive oxygen species (ROS) formation in kidney revealed by increased lipid peroxidation (LPO), decreased GSH in addition to increased inducible nitric oxide synthase (iNOS) level establishing the oxidative stress role in pathogenesis of renal damage, vascular injury, and endothelium dysfunction induced by Cyclosporine A (Hewedy and Mostafa, 2016).
Administration of GSPE to CsA treated rats revealed significant decreases in serum levels of urea and creatinine, similar data were recorded by Yun et al., (2020) who recorded that administration of GSPE exhibited better recovery against renal oxidative damage induced in rats. The improvement in kidney function is related to the antioxidant properties of proanthocyanidins with flavonoid structure that give the ability to decrease blood levels of creatinine and urea by increasing glomerular filtration rate (Nazima et al., 2015).
A significant increase in kidney tissue L-MDA concentration with marked decreases in GSH level and CAT activity were observed in CsA treated rats. The increased lipid peroxidation increases peroxo and hydroxyl radicals resulting in oxidative damage (Mustafa et al., 2018). Our results agreed with Hussein et al., (2014) who demonstrated that a significant increase in renal L-MDA level resulted by CsA treatment to normal rats, suggesting the role of oxygen free radicals involved in renal injury. ROS production by CsA may be due to the drug action as an uncoupler and inhibitor of the mitochondrial electron transport system and metabolism of CsA by cytochrome P450 3A (Ateyey, 2015).
Endogenous antioxidant defense system decreased due to results from ROS production. Excessive ROS decrease the renal antioxidant capacity and make the kidney more susceptible to oxidative damage. Significant decreases in...
Our study showed that CsA treatment induced significant up-regulation of kidney tissue NF-κB and PAI-1 gene expression levels. CsA administration reduced the renal content of GSH, CAT and SOD activities resulted in oxidative damage expressed by increase in lipid peroxidation, iNOS and NF-κB expression (Balah, 2014). The NF-κB transcription factor has a role involved in the transcription of inflammatory iNOS and other inflammatory genes in response to oxidative stress (Aktan, 2004). Plasminogen activator inhibitor-1 (PAI-1) is a pro-fibrotic cytokine considered as the major inhibitor of fibrinolysis (Ghosh and Vaughan, 2012). PAI-1 increased expression in vivo inhibits fibrinolysis, and leading to the pathological fibrin deposition and tissue damage (Aso, 2007). CsA nephrotoxicity mechanism involved endothelial function impairment, vasoconstrictors and vasodilators mediators unbalance, activation of renin-angiotensin-aldosterone system and increased cell proliferation. These mediators increased transforming growth factor-β1 (TGF-β1) expression and plasminogen activator inhibitor-1 (PAI-1) which led to disruption of tissue architecture by increasing extracellular matrix synthesis and decreasing its degradation, resulting in fibrosis of tubule-interstitial and arteriolopathy (Young et al., 1995).

Meanwhile, GSPE administration significantly down-regulated NF-xB and PAI-1 gene expressions in kidney tissues compared with CsA treated rats. Similarly, Hussein et al., (2018) showed that GSPE significantly decreased kidney iNOS, TNF-α, NFκB and caspase-3. GSPE administration declined renal injury markers and oxidative damage, and inactivated the inflammatory pathway by NF-xB inhibition (Wei et al., 2012). Proanthocyanidins have many protective effects including anti-oxidation and anti-inflammation (Limtrakul et al., 2016). Li et al., (2016) indicated that GSPE significantly reduced mRNA expressions of inflammatory cytokine genes including PAI-1 in small intestine. Moreover, Sandra et al., (2010) showed that GSPE down-regulated urokinase-type plasminogen activator and plasminogen activator inhibitor-1.

Cyclosporine A treated rats showed significant up-regulation in kidney tissue gene expressions of apoptotic (caspase-3, p53) and down-regulation of anti-apoptotic (Bcl-2). Renal cell apoptosis and kidney dysfunction were important features of chronic CsA nephrotoxicity (Xiao et al., 2013). The significant up-regulation in kidney tissue p53 gene expression in CsA treated rats came in accordance with (Moon and Kim, 2019). Activation of p53 induced a series of programs, including cell-arrest, cellular senescence and apoptosis (Hsin et al., 2006). Cell apoptosis signaling pathway is activated by the mitochondrial and endoplasmic reticulum (ER) stress. Besides, mitochondrial injury induces mitochondria release of cytochrome C, resulting in mitochondrial transmembrane potential loss (Muthuraman et al., 2011) and activation of caspase family of proteases; caspase-3 and caspase-9 (Liu et al., 2019). The anti-apoptotic Bcl-2 protein is able to inhibition of Bax-induced apoptosis (Rossé et al., 1998). The observed down-regulation in Bcl-2 protein expression came in accordance with Ateyya, (2015).

CsA down-regulated Bcl-2 and Bcl-XL resulted in translocation of Bax to the mitochondria and impaired Bcl-2 and Bax balance in renal tubular cells (Xiao et al., 2013). Conversely, GSPE administration exhibited down-regulation in kidney tissues Caspase-3 and p53 and up-regulation of Bcl-2 gene expression levels. The anti-apoptotic GSPE protective effect by p53 tumor suppressor protein modulation was confirmed by Yousef et al., (2018). GSPE treatment resulted in a gradual decreased in caspase-3 level, this decline in caspase-3 restored the enzyme pathway. The increase in Bcl-2 protein expression was related to presence of both hydrophobic and hydrophilic residues within the flavan-3-ol molecules, giving these molecules the ability to interact with the general structure of BCl-2 family (Nazima et al., 2015).

5. CONCLUSIONS

It could be concluded that, GSPE has a reno-protective effect by enhancing antioxidant defense system, and attenuates CsA induced nephrotoxicity and oxidative stress. Moreover, GSPE has a strong anti-inflammatory effect through modulation of NF-xB, PAI-1 gene expressions, and anti-apoptotic effect by inhibiting caspase-3, p53 and activating Bcl-2 signaling pathways.

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