RESULTS AND DISCUSSION
RESULTS AND DISCUSSION

4.1 ANALYSIS OF ARJUNOLIC ACID

4.1.1 COLUMN CHROMATOGRAPHY

Figure 19:
After eluting 2ml of samples in 10 number of tubes, the presence of triterpenoid were determined by Anisaldehyde sulphuric acid test. The yellowish golden color in 5,6,7,8 number tubes indicates the presence of triterpenoids containing AA. Nearly 0.395g of triterpenoid extract were obtained from 1kg bark powder of Terminalia arjuna and subsequently 0.269g of AA extract was obtained from Column chromatographic technique.
The samples of arjunolic acid were spotted on TLC plate and run with solvent chloroform methanol (2:1). The spots for arjunolic acid were confirmed by Rf values. The chromatogram obtained with test solution shows a band at RF~0.65 corresponding to the reported Rf values of arjunolic acid.
4.1.3 FTIR PATTERN

Figure 22:

FTIR INTERPRETATION

The FTIR pattern shows the IR spectrum of triterpenoids of Terminalia arjuna bark. In broad spectrum between 1320-1000 cm\(^{-1}\) shows the hydroxyl groups (-OH) and carboxylic acid peaks of triterpenoids of Terminalia arjuna (arjunin, arjunic acid, arjunolic acid, arjungenin). A broad peak between 1760-1690 cm\(^{-1}\) represents -OH in plane vibration. This spectrum confirms the structures of triterpenoids present in the extract.
4.1.4 HPLC CHROMATOGRAM

In the chromatogram obtained from the well resolved peak at RF 0.857 was obtained for arjunolic acid. The peak was pure, with no interference from the excipients used in the formulation.
4.1.5 NMR SPECTRAL DATA ANALYSIS

4.1.5.1 $^{13}$C NMR of Arjunolic Acid

Figure 24:

$^{13}$C –NMR (500 MHz, DMSO d$_6$) shows peak at $\delta_{ppm}$ 10.062, 11.028, 13.047, 13.088 correspond to CH$_3$ group of C-24, 18.025, 18.119, 18.533, 18.872 correspond to C-5 and C-25, 22.337, 22.635, 22.794, 23.573, 23.951 correspond to C-19, 25.282, 25.822, 26.732 correspond to C-10, 28.743, 28.852, 28.932, 28.991, correspond to C-27, C-28 & C-29, 29.068, 29.110, 29.199, 29.260, 29.377, 29.432 correspond to C-9, 30.234 correspond to C-14, 31.264, 31.866 correspond to C-16, 33.734 correspond to C-6, 35.044 correspond to C-13, 38.776 correspond to C-21, 47.135, 47.306, 47.477, 47.646, 47.818, 47.987 correspond to C-3, C-4, C-8, C-15 & C-22, 48.158 correspond to C-20, 50.324 correspond to C-11, 67.788, correspond to C-23, 71.059 correspond to C-1, 78.054 correspond to C-2. The peaks at $\delta_{ppm}$ 128.495, 129.478, 131.060 correspond to C-18.
Figure 25:

$^{13}$C NMR of Arjunolic Acid
Figure 26:

$^{13}$C NMR of Arjunolic Acid
Figure 27:

$^{13}$C NMR of Arjunolic Acid
Figure 28:

The $^1$H-NMR (500 MHz, DMSO) gave the signals at 0.74, 0.76, 1.0, 1.5 assigned to six singlet methyl groups, 2.0, 2.14 assigned to one doublet methyl group. The assignments of various signals are: 3.46, 3.59 (glucosidic ring protons), 7.84 (1H).
Figure 29:

$^1$H NMR of Arjunolic Acid
Figure 30:

$^1$H NMR of Arjunolic Acid
Figure 31:

$^1$H NMR of Arjunolic Acid
4.1.6 IR SPECTRAL DATA ANALYSIS OF ARJUNOLIC ACID

Figure 32:

IR analysis indicated the presence of olefinic (1634 cm\(^{-1}\)), hydroxyl (3427 cm\(^{-1}\)), methyl (1384 cm\(^{-1}\)) and carboxylic acid (hydroxyl at 2927 cm\(^{-1}\) and 1103 cm\(^{-1}\)) moieties. There was also a crowded signal indicating the presence of –CH\(_2\), glycosidic and –OH groups (3778 cm\(^{-1}\), 2405 cm\(^{-1}\), 1103 cm\(^{-1}\), 793 cm\(^{-1}\), 667 cm\(^{-1}\), 605 cm\(^{-1}\)).

The triterpenoid skeleton was easily deduced from 1H NMR data by the appearance of vinylic (R2C=CHR, δH 3.59), hydroxylated methine (RCHOH, δH 3.46) and acetyl (CH3COOO, δH 2.00) moieties (Table 1). There was also a crowded signal indicating the presence of –CH3, -CH2, and –CH.
protons (δH0.7,δH 32.97). The 13C NMR spectrum confirmed these findings. In addition it showed the typical carboxyl function at δc 168.005 of the –CO2H constituent. Altogether, eight methyl groups, ten methylenes, eleven methines and nine quaternary carbons were revealed by DEPT data was reported by Jeremiah et al., (2007).

4.2 CHANGES IN BODY WEIGHT AND KIDNEY WEIGHT

The body weight of each animal was recorded and the percentage of weight gain was noted along with changes in kidney to body weight ratio and presented in (Table 2). A significant weight loss and a significant increase (P<0.001) in kidney/body weight ratio was observed as a result of CsA administration compared to the control group. CsA reduces the amount of food intake which results in decreased body weight as observed in previous studies (Wongmekiat et al., 2008). Administration of Arjunolic acid (AA) markedly increased mean final body weight and decreased the mean kidney to body weight ratio (p<0.001). No significant (p<0.001) changes on comparison with control (Group 1) and AA treated groups (Group 4) were observed.

The decrease in the rate of body weight gain in CsA treated animals has been reported by previous studies (Ferguson et al., 1993). Concomitant administration of AA significantly decreased CsA induced weight loss, indicating that oxidative stress might be a factor involved in weight factor. Manna et al., (2009), reported the efficacy of AA against STZ induced diabetic nephropathy in rats.
Table 2: Body Weight gain and changes in kidney body weight ratio of control and experimental animals

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>CsA</th>
<th>CsA + AA</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain%</td>
<td>154.33±14.71</td>
<td>98.33±14.71(^a)</td>
<td>150.16±10.20(^b)</td>
<td>163.66±7.22(^c)</td>
</tr>
<tr>
<td>Kidney/body weight ratio</td>
<td>0.139±0.009</td>
<td>0.125±0.007(^a)</td>
<td>0.137±0.005(^b)</td>
<td>0.134±0.008(^c)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D for six animals in each group
Comparisons are made between Control Vs CsA\(^a\), CsA Vs CsA + AA\(^b\), Control Vs AA\(^c\).

Values were extremely statistically significant for B.Wt.at (P<0.001) and Kidney/B.Wt.at (P<0.05) for a and b, not significant for c.

Table 3: Effects of AA and CsA on Creatinine and Urea Clearance

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>CsA</th>
<th>CsA + AA</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine Clearance</td>
<td>1.53±0.02</td>
<td>1.05±0.06(^a)</td>
<td>1.34±0.02(^b)</td>
<td>1.45±0.08(^c)</td>
</tr>
<tr>
<td>Urea Clearance</td>
<td>1.02±0.01</td>
<td>0.93±0.01(^a)</td>
<td>0.99±0.04(^b)</td>
<td>1.03±0.02(^c)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD; n=6

Creatinine Clearance and Urea Clearance in control and experimental rats.

4.3 BIOCHEMICAL PARAMETERS

In the present study, treatment of rats with cyclosporine for a period of 21 days resulted in significant (p<0.001) increase in serum creatinine, urea
and uric acid level, suggesting significant functional impairment in the kidneys. Earlier studies were reported significant alterations in the level of BUN and creatinine following chronic CsA treatment (Mason, 1990; Tirkey et al., 2005). Treatment of rats with AA alone results in improvement of renal function reflected by significant decrease in blood urea, creatinine and uric acid levels when compared to control rats. When AA was administered to the cyclosporine treated groups, there was a significant improvement in renal function of the rats and the level of urea, uric acid and creatinine decreased significantly (p<0.001) compared to the CsA treatment group.

CsA induces significant decrease in creatinine clearance and increase in urinary protein excretion and marked decrease in serum protein and is prevented by AA co-administration. CsA-dependent hypertension is likely due to vasospasm of the renal microcirculation, resulting in increased renal vascular resistance with concomitant decrease in GFR (Epstein et al., 1998; Kaye et al., 1993; Zhong et al., 1998). These effects may account for the increased serum creatinine and reduced creatinine clearance observed in the present study. Serum creatinine clearance is the most widely accepted method for non-invasive estimation of glomerular filtration rate in clinical practice to diagnose patients with cyclosporine A toxicity (Paul & De Fijter, 2004). Serum urea, uric acid and creatinine levels are conventional indicators of renal injury (Oshida et al., 2008; Faddah et al., 2012).
Table 4: Effects of CsA and AA on Serum Constituents

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>CsA</th>
<th>CsA + AA</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>20.7 ±1.8</td>
<td>51.6 ± 5.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.0 ± 2.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.6 ± 1.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Creatinine</td>
<td>3.36 ± 0.24</td>
<td>6.0 ± 0.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.7 ± 0.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.09 ± 0.31&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Uricacid</td>
<td>4.58 ± 0.14</td>
<td>5.78 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.06 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.35 ± 0.15&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD; n=6

One way ANOVA followed by student’s t test

Units: Urea, Creatinine, Uric acid – mg/ dl serum

Values were extremely statistically significant at (P<0.001) for a and b, not significant for c.

Comparisons were made between Control Vs CsA<sup>a</sup>, CsA Vs CsA + AA<sup>b</sup>, Control Vs AA<sup>c</sup>

4.4 EFFECTS OF CsA AND AA ON RENAL ENZYMIC STATUS

Renal function is highly dependent on mitochondrial energy; hence, the kidneys are susceptible to mitochondrial decay. Recent data suggest that the kidneys are the main targets of mitochondrial impairment at the onset of, as well as throughout, streptozotocin-induced diabetes (Katyare & Satav, 2005). Most studies show the consequences of oxidative stress on mitochondrial function exposed to chemical insults, or by the use of chemical inhibitors of mitochondrial function, results in increased ROS production (Boveris, 1984; Turrens et al., 1985; Martensson et al., 1991; Chinopoulos et al., 1999; Gibson et al., 1999; Nulton-Persson & Szweda, 2001). Genetic
models of oxidative stress are new and represent the consequences of oxidative stress in mitochondrial function via the inactivation of key antioxidant proteins in the mitochondria (Li et al., 1995; Jha et al., 2000; Higgins et al., 2002; Jung et al., 2002).

The pathophysiologic mechanisms of CsA-induced nephrotoxicity have been deeply studied, and several mediators, such as the renin-angiotensin-aldosterone system, the platelet activator factor, endothelin, and eicosanoids have been implicated (Coffman, et al., 1987; Dos Santos et al., 1991; Perico et al., 1986). CsA increases glomerular synthesis and thromboxane urinary excretion in rats, suggesting that this eicosanoid may have a relevant role in renal impairment (Parra et al., 1988a). The ultimate mechanisms by which ROS increase is produced in response to CsA treatment have not been established so far. Since mitochondria constitute one of the main sources of ROS within the cells. The molecular mechanisms by which CsA induces ROS production are not well understood, although it has been considered that their production could be promoted by either the drug’s action on several systems like cytochrome P450, NADPH oxidase or xanthine oxidase, or either the result of decreased cellular antioxidant systems (Jeon et al., 2005).

Since mitochondria are one of the main physiologic sources of ROS, our study was conceived with the aim of defining the possible role of mitochondria in CsA-induced nephrotoxicity. In fact, under normal conditions, it is estimated that 1-2% of the oxygen of the respiratory chain is
turned into $O_2^-$ (Kowaltowski & Vercesi, 1999). However, mitochondria have a complex antioxidant system that can detoxify these ROS (Kowaltowski & Vercesi, 1999), among which there are enzymes (such as dismutase superoxide, glutathione peroxidase, catalase, glutathione reductase or glutathione NADP trans-hydrogenase) and other smaller molecules (such as glutathione, NAD(P)H, and vitamins E and C) that combined constitute an effective antioxidant system (Turrens, 1997). The action of these compounds is integrated so that the $O_2^-$ released in the respiratory chain is metabolized by the mitochondrial dismutase superoxide (SOD-Mn) to hydrogen peroxide, and this, in turn is detoxified by intra- and/or extramitochondrial glutathione peroxidase (Chance et al., 1979; Fridovich, 1997; Radi et al., 1991). Glutathione peroxidase is kept in its reduced state by glutathione, which in turn is reduced by NADH and NAD(P)H by the NADP-trans-hydrogenase (Chance et al., 1979).

Intracellular antioxidant enzymes provide important cellular defense against ROS-induced oxidative damage. SOD and CAT mutually play important role in elimination of ROS. Deviation in the physiological concentration of these enzymes causes drastic effect on the cellular resistance. Marked decrease in the activities of mitochondrial enzymes with its properties including SOD, CAT, GST, GR and GPx, suggesting that the pro-oxidant-antioxidant balance is disturbed leading to oxidative damage (Parra Cid et al., 2003). It is evident that CsA induces oxidative stress, but its origin remains speculative.
The increased production of reactive oxygen species (ROS) and elevated oxidative stress implicates the development and progression of wide range of conditions including diabetes, cardiovascular disease and renal disease (Halliwell, 1991). Cyclosporine A (CsA) improves patient and organ–graft survival rates, its chronic nephrotoxicity is still a concern. CsA increases the production of ROS in animal and in vitro models, leading to increased oxidative stress and is involved in CsA-induced nephrotoxicity. ROS modulates renal haemodynamics and function, leading to vasoconstriction, and induces renal inflammation and tissue growth (Chade et al., 2003). The superoxide anion reacts with nitric oxide, to form peroxynitrite and impairs intra-renal vascular and glomerular function. Mitochondrial antioxidant defense mechanisms are highly exhausted during increased production of free radicals and disrupt mitochondrial membrane (Trushina & Murray, 2007).

Antioxidant supplementation is suggested by many researchers to reduce oxidative stress in observational and animal studies (Rimm et al., 1993; Crawford et al., 1998) and physicians recommended these supplements to patients. Although most, but not all, large-scale prospective clinical trials shows little benefit with antioxidant supplementation and these supplements provides beneficial role during elevated oxidative stress or compromised antioxidant defences (Jialal & Devaraj, 2003). Meena et al., (2013), have reported the clinical study of arjuna (Terminalia arjuna) Twak Churna on Madhumeha.
Terminalia arjuna is versatile traditional medicinal plant. The experimental studies revealed that its bark showed significant antioxidant (Vaidya et al., 2008), antidiabetic (Raghavan & Kumari, 2006), antigastric ulcer (Devi et al., 2007), antimutagenic (Vaidya et al., 2008), anthelmintic, (Bachaya et al., 2009) activities. Shikha et al., (2013) reported the antifungal activity of alcoholic leaf extracts of Terminalia Catappa and Terminalia Arjuna on some pathogenic and allergenic fungi. It improves the blood circulation to heart, regulate blood pressure (Nammi et al., 2003) and is used for treatment of hypercholesterolemia (Tiwari et al., 1990; Ram et al., 1997; Chander et al., 2004), inhibits platelet aggregation (Namita et al., 2009). Recently reported the evaluation of Terminalia arjuna on cardiovascular parameters and platelet aggregation in patients with Type II diabetes mellitus (Usharani et al., 2013). It protects liver and kidney against the harmful effect of free radicals. Terminalia arjuna showed high potency against drug resistant/clinically studied microbes. Little work has been done in some of the biological activities like immunomodulatory activity. It has been reported that aqueous extract of Terminalia arjuna bark decreases SGPT, ALP & TBARS and increases SOD, CAT, GSH compared to vitamin C (Manna et al., 2006). Co-supplementation of AA, in the present study, significantly (p<0.001) increased the tissue level of enzymatic antioxidants compared to CsA treated rats. This might indicate the usefulness of AA, as an excellent source of antioxidant, in modulating CsA induced nephrotoxicity. T. arjuna extract can deplete the GSH levels and promote oxidation induction, which switches the mode of death via apoptosis (Sivalokanathan et al., 2006).
Figure 33 shows the activity of enzymic antioxidants in the kidney mitochondria of control and experimental group of animals. The activities of enzymic antioxidants were significantly (p<0.001) reduced in CsA induced rats (Group 2) as compared to control animals (Group 1). AA administration (Group 3) significantly increased the enzymic antioxidant activity as compared with CsA induced rats (Group 2). No significant changes were observed in AA alone treated group of rats (Group 4) when compared to control.

**Figure 33: Effect of CsA and AA on the activity of Kidney mitochondrial Enzymic antioxidants**

Values are expressed as mean ± SD for six animals in each Group.

*One way ANOVA followed by student's t test using SPSS16 software package*

Units: **SOD**- 50% inhibition of epinephrine auto oxidation/min/mg protein  
**CAT**- µ moles of H₂O₂ decomposed/min/mg protein  
**GPx**- µ moles of GSH oxidized/min/mg protein
**4.5 STUDY ON THE EFFECT OF AA ON THE LEVEL OF OXIDANTS AND NON ENZYMIC ANTIOXIDANTS OF CsA INDUCED NEPHROTOXICTY**

The membrane polyunsaturated fatty acid (PUFA), are highly susceptible to free radicals induced oxidative damage. Interaction of reactive oxygen radicals with PUFA initiates self propagating lipid peroxidation reactions (Pepicelli et al., 2005) resulting in impaired function, structural integrity and inactivates several membrane bound enzymes (Halliwell et al., 1999). It has been found that CsA treatment increases lipid peroxidation in the mitochondria of kidney (as indicated by the increased level of MDA, end product of lipid peroxidation). CsA induced lipid peroxidation causes degradation of phospholipids and results in cellular deterioration in the mitochondrial kidney. Kidney mitochondria play an important role in maintaining the cellular integrity of kidney and helpful to prevent CsA induced mitochondrial membrane damage by oxidative stress. Hence there is a leakage of mitochondrial enzymes. Treatment with AA along with CsA administration prevented the enhancement of lipid peroxidation and the kidney is maintained significantly.
Chronic administration of CsA produces oxidative stress and increases lipid peroxidation in kidneys by renal TBARS levels. Oxidative stress promotes the formation of vasoactive mediators, affects renal function directly by causing renal vasoconstriction or decreases glomerular capillary ultrafiltration coefficient; and reduces glomerular filtration rate (Garcia-Cohen et al., 2000). The attenuation of lipid peroxidation in CsA-treated rats by CMN provides evidence for the involvement of ROS in CsA-induced lipid peroxidation. It has been suggested that ROS attack unsaturated bonds of membrane lipids in autocatalytic process and results in lipid peroxidation. The oxidative breakdown of membrane polyunsaturated fatty acids causes increased cellular membrane permeability with subsequent alterations of ionic gradients and disruption of several membrane functions and metabolic processes (Amudha et al., 2006). The increased levels of ROS and lipid peroxidation products following CsA have been reported in many experimental invitro and invivo studies (Amudha et al., 2006; Anjaneyulu et al., 2003; Inselmann et al., 1990; Tariq et al., 1999; Zhong et al., 2001).

Reactive oxygen species contribute to CsA-induced hypertension and nephrotoxicity by different mechanisms like, inactivation of NO, a direct vasopressor effect and formation of vasoconstrictive arachidonic acid peroxidation products which reduces renal blood flow along with reduced creatinine clearance (Navarro-Antolin et al., 1998; Vaziri et al., 2002). Nitric oxide possesses physiological and pathological processes (Gossmann et al., 2001). In the present study, a decline in NO levels was observed in rats treated with CsA. These results are in agreement with those of other studies in experimental animals (Shihab et al., 2003) and humans (Gossmann et al., 2001). Nitric oxide may react with superoxide radical and form peroxynitrite.
and is capable of nitrating the tyrosine residues of proteins and enzymes (Vaziri et al., 2002) leading to tissue injury.

NO is important messenger molecule involved in both beneficial and harmful processes within the mammalian body (Ray et al., 2007). NO and the expression of endothelial (eNOS) and inducible (iNOS) isoforms of NOS were recognized as important mediators of physiological and pathological processes of renal injuries like renal ischemia/reperfusion (Vinas et al., 2006), partial or complete unilateral ureteral obstruction (Yoo et al., 2010), chronic renal disease (Nakayama et al., 2009) and hydronephrosis (Kipari et al., 2006). Peroxynitrite anions are generated by the reaction of nitric oxide with superoxide anion. These peroxynitrite anions oxidize biomolecules and promote lipid peroxidation and tubular cell damage (Stamler et al., 1992). Increased nitric oxide production causes depletion of cellular ATP and inactivates enzymes containing iron-sulfur clusters involved in mitochondrial electron transport (Rivas-Cabanero et al., 1997). Nitrosylation of sulfhydryl groups or tyrosine residues in proteins impairs functional properties of these proteins.

Nitric oxide damages DNA, and stimulates DNA repair enzyme poly-ADP-ribose synthetase (Dawson et al., 1992). Studies done by Amore and colleagues demonstrate that CsA induces apoptosis in various renal cell lines, and this effect is mediated by the induction of iNOS (Amore et al., 2000).

Endothelial NO production is the basis of vasodilatation. Recent studies show the role of NO in the pathophysiology of acute renal failure. Peresleni et al. (1996) showed that oxidative stress in epithelial cells reduces
cell life by increasing NO and nitrite levels (Peresleni et al., 1996; Perez de Hornedo et al., 2007). Reduced glutathione is the sulphur-containing nucleophilic substance found in kidney. It plays important role in protecting the cells against oxidative stress and detoxification of xenobiotics, including CsA (Ketter et al., 1983). In glutathione redox cycle, GSH together with GPX, converts lipid peroxides to non-toxic products (Ketter et al., 1983) and thereby maintaining the integrity of the mitochondria and cell membranes. The depletion of GSH stores and reduction in GPX activity in the kidney of present study is consistent with other previous investigations (Tariq et al., 1999; Al Khader et al., 1996).

Depletion of renal GSH stores by CsA in the present study promotes inhibition of renal GPX activity. Vaziri et al., (2000) shows induced hypertension in normal rats by glutathione depletion. Another study by Inselmann et al., (1994) reported enhancement of CsA-induced nephrotoxicity by glutathione depletion. Data obtained from the present study support the link between oxidative stress, hypertension and nephrotoxicity. A similar correlation has been shown by other studies (Parra et al., 1998; Nishiyama et al., 2003; Mohamadin et al., 2005). It has already reported that AA effectively reduces these alterations against myocardial necrosis (Sumitra et al., 2001). Recent data showed that AA had free radical scavenging activity in a cell free system and it exhibits antioxidant power in vivo (Manna et al., 2008). In a study by Ali et al., (2003) arjunapthanoloside isolated from the stem bark of terminalia arjuna showed a potent antioxidant activity and inhibited nitric oxide production in lipopolysaccharide stimulated rat peritoneal macrophages. Alam et al., (2008), in their study has shown that oleanane-type triterpene glycosides designated as Termiarjunoside I and Termiarjunoside II isolated
from stem bark of Terminalia arjuna, potently suppressed the release of nitric oxide and superoxide from macrophages and also inhibited aggregation of platelets.

Table 5 shows the effect of CsA and AA on the levels of LPO, Nitrite and GSH in the kidney mitochondrial tissue of control and experimental animals. Tissue LPO levels were significantly increased in CsA administered group (Group 2), whereas Nitrite and GSH levels found to be decreased in CsA treated rats. AA supplementation (Group 3) significantly (p<0.001) decreased the concentration of LPO and increased the concentration of Nitrite and GSH levels as compared to CsA induced rats (Group 2). No significant difference was found between control group (Group 1) and AA alone treated animals (Group 4). The enhancement of antioxidant defence by AA possess major role to decrease the toxicity in this model which has been esstablished well in other models.

Table 5: Effect of AA and CsA on the levels of oxidants and antioxidants in the Kidney mitochondria of control and experimental group of animals

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>CsA</th>
<th>CsA+ AA</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPO</td>
<td>3.38 ±0.18</td>
<td>5.20 ± 0.15a</td>
<td>4.51 ± 0.13b</td>
<td>3.21 ± 0.20c</td>
</tr>
<tr>
<td>Nitrite</td>
<td>0.89 ± 0.08</td>
<td>0.30 ± 0.05a</td>
<td>0.66 ± 0.09b</td>
<td>0.84 ± 0.05c</td>
</tr>
<tr>
<td>Protein</td>
<td>0.29 ± 0.02</td>
<td>0.50 ± 0.06a</td>
<td>0.37 ± 0.02b</td>
<td>0.27 ± 0.08c</td>
</tr>
<tr>
<td>GSH</td>
<td>34.6 ± 3.72</td>
<td>15.0 ± 1.29a</td>
<td>35.67 ± 4.97b</td>
<td>34.0 ± 3.58c</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD for six animals in each Group.
One way ANOVA followed by student’s t test

Units: LPO – nmoles of MDA released/mg of protein
Nitrite-mg of Nitrite/mg of protein/100ml of tissue
Protein: mg/g of tissue

GSH- n moles / g tissue

Values were extremely statistically significant at (P<0.001) for a and b, c- not significant.

Comparisons are made between Control Vs CsA^a, CsA Vs CsA + AA^b, Control Vs AA^c

4.6 STUDY OF MITOCHONDRIAL ENZYMES

Mitochondria, the garden of cell death, play an important role in regulating cell death pathways (Green & Reed, 1998; Green & Kroemer, 2004). ROS predominantly occurs in mitochondria and possess important role in apoptosis. Mitochondria are the important cell organelles in research due to its regulatory role in energy balance (Wallace, 1999). Various NAD/NADP-linked enzymes are intricately involved in the maintenance of the reduced redox state in mitochondria thereby providing reducing power to generate ATP via oxidative phosphorylation (Lee & Lardy, 1965; Maechler & Wollheim, 2001). Cyclosporine causes imbalance in the cellular oxidative stress due to its excessive ROS formation. The CsA is to be an uncoupler and inhibitor of the mitochondrial electron transport system, its action on NADPH oxidase or xanthine oxidase, results in decreased cellular antioxidant system or the CsA metabolism of cytochrome P450 (Jeon, 2005). ROS attack unsaturated bonds of membrane lipids in autocatalytic process and results in lipid peroxidation.

The main mechanisms responsible for mitochondrial ROS production are the respiratory chain, in particular its complexes I and III (Beyer, 1992; Cadenas & Davies, 2000; Raha & Robinson, 2000), in the inner mitochondrial membrane, and monoamine oxidase in the outer membrane. ROS accumulates
and causes damaging effects in cell and the whole organism (Halliwell & Gutteridge, 1989; 1990; Mizuno et al., 1998; Schapira, 1999; Cadenas & Davies, 2000; Raha & Robinson, 2000). Mitochondria contribute in human metabolism, includes oxidative phosphorylation, the tricarboxylic acid (TCA) cycle, fatty acid oxidation, iron sulfur center and heme biosynthesis and amino acid metabolism (Wallace, 2010; Duchen & Szabadkai, 2010; Murphy, 2009; Smith et al., 2010). Mitochondria are central to apoptotic cell death and modulate calcium fluxes throughout the cell (Wallace, 2010; Mammucari, 2011). Superoxide is a reactive oxygen species (ROS) produced by mitochondria, and it underlies redox signaling in hypoxia sensing, cell differentiation and innate immunity (Murphy et al., 2009; Arnoult et al., 2011; Tormos et al., 2011). Oxidative damage to mitochondria disrupts the function of the organelle making cell death more probable and contributes to diverse pathologies like sepsis, organ deterioration in transplantation, I/R injury, diabetic complications and also neurodegenerative diseases (Wallace, 2010; Murphy, 2009).

Mitochondrial ATP synthesis is frequently disrupted by damage to the respiratory chain, the inner membrane or the ATP synthesis machinery, thereby contributing to cell death and dysfunction (Wallace et al., 2010). Recent study showed that Cyclosporine A induces apoptosis in renal tubular cells is related to oxidative damage and mitochondrial fission (De Arriba et al., 2013). Triterpenoids, flavonoids, tannins and phytosteroids possess significant (P<0.05) antioxidant antihyperglycemic (Ragavan & Krishnakumari, 2006) and cardioprotective effect (Khanna, 1996). Manna et al., (2008) have reported that AA is a potent antioxidant and free radical
scavenger and protect the cells from metal induced toxicity (Manna et al., 2008).

Figure 34 shows the activities of the mitochondrial enzymes NADH DH, α-kG DH and ICDH in the kidney of control and experimental group of animals. The activities of mitochondrial enzymes showed significant decrease (p<0.001) in CsA induced rats. Treatment with AA altered the activities of these enzymes to near normal levels, when compared with CsA induced animals. This is agreeable with recent studies where the alteration in enzyme mitochondrial complex activities were significantly restored by abana containing Terminalia arjuna (Tandon et al., 1995).

Figure 34: Effects of AA and CsA on the activity of mitochondrial enzymes in the kidney of control and experimental group of animals.

![Graph showing enzyme activities](image)

Values are expressed as mean ± SD for six animals in each group

One way ANOVA followed by student’s t test using SPSS16 software package

Units: NADH DH: nmoles of NADH oxidized/min/mg protein
α-KGDH: nmoles of ferrocyanide liberated/min/mg of protein

ICDH: nanomoles of α-Ketoglutarate liberated/min/mg protein

Values were extremely statistically significant at \((P<0.001)\) for \(a\) and \(b\), \(c\)-not significant.

Comparisons are made between Control Vs CsA\(^a\), CsA Vs CsA + AA\(^b\), Control Vs AA\(^c\)

4.7 STUDY OF MITOCHONDRIAL ENZYMES IN CYTOSOL

When the level of ROS exceeds the capacity of the mitochondria and cell to detoxify them, the resulting chronic oxidative stress activates mitochondrial permeability transition pore (mtPTP) (Petronilli et al., 1994). The activation of mtPTP creates an open channel across the mitochondrial inner and outer membranes, which permits the free diffusion of molecules of less than 1,500 Da between the matrix and the cytosol (Zoratti & Szabo, 1995). This results in the collapse of DC, the loss of matrix solutes swelling of mitochondria and releases cytochrome c, procaspases 2, 3, and 9, apoptosis-initiating factor, and caspase-activated DNAse. Cytochrome c and the cytosolic factor Apaf1 which activate caspases and degrade cytosolic proteins, while apoptosis initiating factor and caspase-activated DNase move to the nucleus and degrade the chromatin (Liu et al., 1996). Thus mtPTP regulates programmed cell death or apoptosis.

During oxidative stress, mtPTP were activated by decreased mitochondrial DC, reduced levels of matrix ADP/ATP ratio and uptake of excessive \(\text{Ca}^{2+}\) (Bernardi, 1992; Petronilli et al., 1994; Lapidus & Sokolove,
Therefore, decreased mitochondrial energy production and increased mitochondrial oxidative stress initiates apoptosis, resulting in tissue decline and senescence. The opening of the mtPTP causes loss of matrix NAD, NADH, stalling the tricarboxylic acid cycle and ETC and blocking coupled respiration. The same pattern of respiratory dysfunction has been observed in the senescence-accelerated mouse (Nakahara et al., 1998), suggesting that the biochemical defect in this animal might be related to mitochondrial energy deficiency, oxidative stress, and apoptosis. Mitochondrial ROS production and oxidative stress causes increased proton leak across the mitochondrial inner membrane, inhibition of the ETC, oxidation of mitochondrial lipids, sensitization of the mtPTP, and ultimately cellular death by apoptosis. The enhancement of antioxidant defence by AA has also played a major role to decrease the oxidative stress in this model. Sinha et al., (2008) have reported the efficacy of AA against arsenic induced nephrotoxicity in mouse model. Recently reported that Terminalia arjuna’s antioxidant effect in isolated perfused kidney (David Raj et al., 2012).

Figure 35 shows the activities of the mitochondrial enzymes NADH DH, α-kG DH and ICDH in the kidney cytosol of control and experimental group of animals. The activities of mitochondrial enzymes showed significant decrease (p<0.001) in group 2 rats. Treatment with AA altered the activities of these enzymes to near normal levels, when compared with CsA induced animals.
Figure 35: Effects of AA and CsA on the activity of mitochondrial enzymes in the kidney Cytosol of (without mitochondria) Control and experimental group of animals.

Values are expressed as mean ± S.D for six animals in each group

One way ANOVA followed by student’s t test using SPSS16 software package

Units: NADH DH – nmoles of NADH oxidized/min/mg protein

αKG DH – nmoles of ferrocyanide liberated/min/mg protein

ICDH – nmoles of α-ketoglutarate liberated/min/mg of protein

Values were statistically significant at (P<0.05) for a and b, c- not significant.

Comparisons: \textsuperscript{a}Control Vs CsA, \textsuperscript{b}CsA Vs CsA + AA, \textsuperscript{c}Control Vs AA.
4.8 STUDY OF TCA CYCLE ENZYMES AND ELECTRON TRANSPORT CHAIN COMPLEXES IN MITOCHONDRIA

Cyclosporine has greatly improved morbidity and mortality in transplantation patients over the past decade and has been increasingly applied with considerable clinical benefit in the treatment of autoimmune diseases (Sigal & Dumont, 1993). Its use is often accompanied by unwanted side-effects, such as hypertension and the deterioration of renal function (Mange et al., 2000; Curtis, 1998). Previous studies have proposed that ROS production and oxidative stress may contribute to CsA toxicity in many tissues (Sander et al., 1996; Parra et al., 1998). The present study investigated the effect of AA on CsA-induced nephrotoxicity. The TCA cycle and electron transport chain are the important determinants of mitochondrial function. In the mitochondrial membrane, unsaturated fatty acid is the component of phospholipid, is being susceptible to oxidation by the hydroxyl radical. The mitochondrial TCA cycle enzymes aconitase, succinate dehydrogenase (SDH), and KGDHC (α-Keto glutarate dehydrogenase) itself are sensitive to oxidative inactivation both in vitro and in vivo (Tretter & Adam-Vizi, 2000; Gibson et al., 2002; Sadek et al., 2002).

Cytosolic and mitochondrial enzymes such as, G6PD, LDH, SDH, MDH and GDH plays important role in the maintenance of physiological conditions in the cell. Fluctuations in these enzyme activities during pathological conditions like diabetes leads to severe physiological malfunctions in the kidney (Boquist et al., 1985). SDH, a marker enzyme for
mitochondria, possess increased activity than other enzymes in developing and adult animals. Decreased SDH activity in diabetic condition affects succinate-fumarate conversion and depresses oxidative metabolism in mitochondria. It has been suggested that the diabetogenicity of STZ, depends on the inhibitory activity of citric acid cycle enzymes like SDH (Boquist et al., 1985, Sener et al., 1990). MDH plays important role in the TCA cycle as SDH. Remarkable decrease in renal MDH activity in diabetic rats indicates irregularity in the TCA cycle and ultimately affects other mitochondrial enzymes. Decreased MDH activity in diabetic rats was also reported by Pannerselvam & Govindaswamy, (2002).

Cyclosporine inhibits the Krebs’ cycle (decreased glutamate/glutamine concentrations) and mitochondrial oxidative phosphorylation (decreased concentrations of NAD$^{+}$), resulting in a significant cellular reduction in ATP and phosphocreatine with a concomitant increase of ADP concentration. The inhibition of mitochondrial energy production caused lactate accumulation followed by elevated fatty acid oxidation at the high cyclosporine doses. The increased lactate concentrations in the cyclosporine-treated rats can be explained by compensatory stimulation of anaerobic ATP synthesis via glycolysis (Schlant, 1978). The biochemical depression of mitochondrial glucose and high-energy phosphate metabolism are very similar to those induced by cyclosporine in the brain (Serkova et al., 2001; Serkova et al., 2002). The ability of cyclosporine to decrease the energy state by inhibiting mitochondrial metabolic pathways was reported for the kidney
also (Henke et al., 1992). Singh & Khare, (2013) were suggested the role of Cyclosporine and mitochondria. Recently reported that Membrane Localized Iridium (III) Complex Induces Endoplasmic Reticulum Stress and Mitochondria-Mediated Apoptosis in Human Cancer Cells (Cao et al., 2013).

The electron transport system consists of four main complexes and several ancillary components bringing about the oxidation of NADH and other substrates by molecular oxygen. These complexes are embedded in the lipid bilayer of the mitochondrial inner membrane (Hatefi, 1985). The flow of electrons through complexes I, III and IV results in extrusion of protons from the matrix side of the inner membrane to the inter membrane space with production of an electrochemical $\text{H}^+$ gradient. The ETC complex subunits possess specific targets for ROS-mediated oxidative modifications. Mitochondria isolated from Type I Diabetic rats showed decreased respiratory chain activity characterized by decreased membrane potential, decreased expression of oxidative phosphorylation genes, and respiratory ratios. Complex I of the mitochondrial electron transport chain is the major site of mitochondrial ROS production (Barja, 1999; Herrero & Barja, 2000; Lenaz, 2001; Sipos et al., 2003).

Several research groups have demonstrated that complex I generate ROS when reduced with NADH, because of no consensus about the site of ROS production in complex I (Lenaz, 2001; Kushnareva et al., 2002; Liu et al., 2002). The mRNAs for Complex I, II, III, and IV were also found to be decreased (Rolo & Palmeira, 2006). Munusamy et al., (2009) reported kidney
mitochondrial impairment in diabetes. The decline in renal mitochondrial respiration is due to increased ROS production at complexes I, III, and IV (Munusamy et al., 2009). AA normalized the activities of the complexes and decreased the production of superoxide due to its radical scavenging activity, which may be attributed to the triterpenoids and other constituents present in the drug. Previous studies reported that AA protects both intrinsic and extrinsic pathways against cadmium induced hepatotoxicity (Pal et al., 2011).

Figure 36 & 37 shows the activities of TCA cycle enzymes and Electron Transport chain complexes in the kidney mitochondria of control and CsA induced experimental group of rats. The Group 2 rats induced with CsA showed considerable impairment (P<0.05) in the activities of TCA cycle enzymes such as SDH, MDH, Aconitase, Citrate synthase and Complexes of I,II,III & IV when compared with Group 1 control rats. However, these severe alterations in the drug metabolizing enzymes were appreciably prevented (P<0.05) in Group 3 rats, treated with AA when compared to induced animals. Previous studies suggested that chemopreventive role of AA against arsenic induced testicular toxicity due to its intrinsic antioxidant property (Manna et al., 2008).
Figure 36: Effects of CsA and AA on the activity of TCA cycle enzymes in kidney mitochondrial homogenate

Values are expressed as mean ± S.D for six animals in each group.

One way ANOVA followed by student’s t test using SPSS16 software package.

Units: SDH: nmoles of succinate oxidized/min/mg protein

MDH: nmoles of NADH oxidized/min/mg protein

Aconitase: nmoles of cis-aconitate formed/min/mg protein

Citrate synthase: nmoles of DNBA formed/min/mg protein

Values were statistically significant at (P<0.05) for a and b, c- not significant.

Comparisons: *Control Vs CsA, †CsA Vs CsA + AA, ‡Control Vs AA.
Figure 37: Effects of CsA and AA on the activity of electron transport chain complexes in the kidney mitochondrial homogenate

Values are expressed as mean ± S.D for six animals in each group

One way ANOVA followed by student's t test using SPSS16 software package

Units: Complex-I – μmoles of NADH oxidized/min/mg protein

Complex-II – nmoles of DCIP reduced/min/mg protein

Complex-III – nmoles of cytochrome c reduced/min/mg protein

Complex-IV – μmoles of cytochrome c oxidized/min/mg protein

Values were statistically significant at (P<0.05) for a and b, c- not significant.

Comparisons: aControl Vs CsA, bCsA Vs CsA + AA, cControl Vs AA
4.9 STUDY OF LYSOSOMAL ENZYMES

Lysosomes are cytoplasmic organelles. Lysosomal enzymes are ubiquitous, biologically active molecules and degrade macromolecules like proteins, carbohydrates, nucleic acids, lipids, and their conjugates. It is well known that it implicates its role in tissue injury and repair, inflammation, and phagocytosis (Gay et al., 1980). Lysosomal-associated membrane proteins are transmembrane lysosomal glycoproteins, detectable at the cell surface of lymphocytes. Undigested oligosaccharides not only accumulate in the lysosomes, but also leak into the cytoplasm, into the circulation and into the extracellular space (Gallin et al., 1988).

Changes in lysosomal enzyme activities may result in impairment of phagocytic and endocytic activities, inadequate extracellular matrix turnover, and remodeling, which suggest that lysosomal enzyme activities might be involved in the pathogenesis of autoimmune diseases (Malech & Gallin, 1987; Winchester, 2001; Gallin et al., 1988). Therefore, we supposed that activities of lysosomal enzymes might also show correlation with ophthalmic signs of the disease. Recently reported the role of lysosomal enzymes in the pathogenesis of liver and kidney injury induced by short and long term administration of some NSAID Drugs in rats (Salah-Eldin et al., 2012).

Lysosomes, which were first described by de Duve and colleagues in 1955 (de Duve et al., 1955) are acidic, single-membrane bound organelles,
present in all eukaryotic cells (de Duve, 1983). The primary function of lysosomes is degradation of macromolecules. Lysosomal hydrolytic enzymes were initially thought to inevitably trigger necrotic cell death, when released into the cytosol (de Duve, 1959). It has been reported that aspartic protease cathepsin D was redistributed from lysosomes to the cytosol upon oxidative stress-induced apoptotic cell death (Roberg & Ilinger, 1988).

Apoptosis is often associated with release of cathepsins into the cytosol, and in addition to cathepsin D, cysteine cathepsins B and L were involved in apoptosis signaling (Boya & Kroemer, 2008; Chwieralski et al., 2006; Guicciardi et al., 2004; Stoka et al., 2007; Kirkegaard & Jaattela, 2009). Moreover, lysosomal destabilization, which will subsequently be referred to as lysosomal membrane permeabilization (LMP), occurs during cell death types like necroptosis and autophagic cell death (Kroemer & Jaattela, 2005; Zhang et al., 2009). LMP is triggered by apoptotic stimuli, includes death receptor activation, endoplasmic reticulum stress, proteasome inhibition, oxidative stress, DNA damage, osmotic stress, and growth factor starvation (Boya & Kroemer, 2008; Chwieralski et al., 2006; Guicciardi et al., 2004; Stoka, et al., 2007; Kirkegaard & Jaattela, 2009).

Increased production of ROS causes destabilization of the lysosomal membrane via massive peroxidation of membrane lipids. Photosensitizers located in lysosomes generate singlet oxygen upon radiation and causes rapid
release of cathepsins to the cytosol (Caruso et al., 2005; Kessel et al., 2000; Reiners et al., 2002). Markers of tubular dysfunction like urinary N-acetyl β-D-glucosaminidase, which is a lysosomal enzyme that originates from proximal tubular microsomes, associated with reduced GFR induced by cyclosporine A (Burdmann et al., 1994; Yamauchi et al., 1998). Previous studies indicated that AA effectively reduces the leakage of lysosomal enzymes into cytosol due to its free radical scavenging activity and cellular antioxidant capability (Manna et al., 2007).

Figure 38 shows the activities of lysosomal enzymes in induced and experimental group of animals. There was a significant increase (P<0.05) in the activities of lysosomal enzymes such as Cathepsin-D, β-D-Glucuronidase, β-D-Galactosidase, β-D-N-acetylglucoaminidase in CsA induced rats when compared with control rats. Group III AA administered rats showed significant (P<0.05) decrease in the activity of lysosomal enzymes when compared to CsA treated rats. No significant difference was observed between control and AA alone treated rats.
Values are expressed as mean ± S.D for six animals in each group

One way ANOVA followed by student’s t test using SPSS16 software package

**Units**:
- Cathepsin-D - µmole of tyrosine liberated/hr/100mg protein
- β–D Glucuronidase: µmoles of p-nitrophenol liberated/min/mg protein
- β –D Galactosidase - µmoles of p-nitrophenol liberated/min/mg protein
- β –D-N-acetyl glucosaminidase - µmoles of p-nitrophenol liberated/min/mg protein

Values were statistically significant at (P<0.05) for a and b, c- not significant.

Comparisons: ^aControl Vs CsA, ^bCsA Vs CsA + AA, ^cControl Vs AA.
4.10 STUDIES ON LIPID PARAMETERS

CsA is very lipophilic agent, facilitates its attachment to cellular membranes. Organelle membranes especially endoplasmic reticulum has large amounts of unsaturated fatty acids and have very large total surface area. This property has been susceptible to oxidative stress related to CsA (Wang & Salahudeen, 1994). Haynes et al., (1985), suggested that both the immunosuppressor role and the toxicity of CsA were related to its hydrophobic property which facilitates its solubilization within the lipid bilayer, diminishing membrane fluidity. This leads to a reduction in LDL clearance in the hepatocyte, increasing the plasma LDL levels and predisposing the individual to the development of atherosclerosis (Stamler et al., 1988; Ferns et al., 1990). Ferns et al., (1990) observed that CsA has a cytopathic effect on the cells of the macrovascular tissue. CsA appears to provoke changes in lipid metabolism (Caltran et al., 1979; Hess et al., 1987; Lowry et al., 1987; Jevnikar et al., 1988; Stamler et al., 1988; Lopez-Miranda et al., 1989).

The membrane polyunsaturated fatty acids (PUFA) are very susceptible to free radicals induced oxidative damage. Interaction of reactive oxygen radicals with PUFA initiates the self propagating lipid peroxidation reactions (Pepicelli et al., 2005) resulting in impaired function, structural integrity as well as inactivation of a several membrane bound enzymes (Halliwell et al., 1999). We observed that CsA treatment enhanced the lipid peroxidation in the mitochondrial kidney (as indicated by the increased level of MDA, end product of lipid peroxidation). Thus, CsA induced lipid
peroxidation leads to the degradation of phospholipids and finally results in cellular deterioration in mitochondrial kidney. Kidney mitochondria play an important role in maintaining the cellular integrity of kidney. CsA promotes mitochondrial membrane damage by oxidative stress. Hence there is a leakage of mitochondrial enzymes. Treatment with AA along with CsA administration prevented the enhancement of lipid peroxidation and the kidney is maintained significantly.

The lysosomal membrane composition plays an important role in the maintenance of lysosomal integrity. Damage to lysosomal membrane components or changes in the membrane structure and fluidity could result in lysosomal destabilization. PLA2 (phospholipase A2), phospholipase C, and sphingomyelinase, enzymes regulate the membrane lipid composition, all show increased activity in the presence of high cytosolic [Ca$^{2+}$] showed the characteristic of apoptosis. Studies using isolated rat lysosomes have shown that these enzymes (Zhao et al., 2005; Wang et al., 2006a; Wang et al., 2006b; Wang et al., 2006c) as well as the phospholipid products arachidonic acid (Zhang et al., 2006), lysophosphatidylcholine (Hu et al., 2007), and phosphatidic acid (Yi et al., 2006), affect the osmotic sensitivity of lysosomes, making them more susceptible to osmotic stress. Cholesterol plays an important role in the maintenance of lysosomal membrane stability. It has been known that addition of cholesterol to lysosomes reduces their permeability (Fouchier et al., 1983). This reduced lysosomal membrane cholesterol level is associated with increased permeability to protons and potassium ions; this, in turn, causes an osmotic imbalance and destabilization.
of the lysosomal membrane (Deng et al., 2009; Jadot et al., 2001; Hao et al., 2008). In the present study, CsA promotes lysosomal membrane damage by oxidative stress. Hence there is a leakage of lysosomal lipids to cytosol. Treatment with AA along with CsA administration prevented the enhancement of lipid peroxidation and the kidney is maintained significantly. Ram et al., (1997b) reported that the ethanolic extract of Terminalia arjuna bark effectively reduces total and LDL cholesterol in hypercholesterolaemic rabbits. It is also been reported that AA has a significant hypocholesterolaemic effect (Gupta et al., 2001).

**Table 6: Effect of AA and CsA on the level of lipids in the kidney mitochondria of control and experimental group of animals.**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>CsA</th>
<th>CsA+AA</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>0.29 ±0.05</td>
<td>0.71 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.51 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.24 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>FFA</td>
<td>0.47 ± 0.05</td>
<td>0.71 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.45 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.27 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>TG</td>
<td>0.48 ± 0.05</td>
<td>0.96 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.60 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.32 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>0.22 ±0.01</td>
<td>0.59 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.44 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.25 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Values are expressed as mean ± SD for six animals in each Group.*

*One way ANOVA followed by student’s t test*

**Units:** Cholesterol, FFA, Phospholipids & Triglycerides –mg /g tissue

*Values were statistically significant at (P<0.001) for a and b, c- not significant.*

*Comparisons are made between Control Vs CsA<sup>a</sup>, CsA Vs CsA + AA<sup>b</sup>, Control Vs AA<sup>c</sup>.*
Table 7: Effects of CsA and AA on the level of cytosolic lipids in the kidney mitochondria of control and experimental group of animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cholesterol</th>
<th>Triglycerides</th>
<th>Free fattyacid</th>
<th>Phospholipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.51 ± 0.05</td>
<td>1.04 ± 0.24</td>
<td>0.27 ± 0.05</td>
<td>0.03 ± 0.003</td>
</tr>
<tr>
<td>CsA</td>
<td>0.81 ± 0.12a</td>
<td>1.43 ± 0.11a</td>
<td>0.47 ± 0.09a</td>
<td>0.05 ± 0.003a</td>
</tr>
<tr>
<td>CsA+AA</td>
<td>0.38 ± 0.07b</td>
<td>0.79 ± 0.09b</td>
<td>0.18 ± 0.03b</td>
<td>0.04 ± 0.006b</td>
</tr>
<tr>
<td>AA</td>
<td>0.51 ± 0.05c</td>
<td>0.91 ± 0.14c</td>
<td>0.31 ± 0.06c</td>
<td>0.03 ± 0.001c</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D for six animals in each group
One way ANOVA followed by student's t test
Units: Cholesterol, TG, FFA, Phospholipids – mg/g tissue
Values were statistically significant at (P<0.05) for a and b, c- not significant.
Comparisons: aControl Vs CsA, bCsA Vs CsA + AA, cControl Vs AA.

Table 8: Effects of CsA and AA on the level of lipids in the kidney lysosomes of control and experimental group of animals in cytosol

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cholesterol</th>
<th>Triglycerides</th>
<th>Free fattyacid</th>
<th>Phospholipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.99 ± 0.14</td>
<td>0.92 ± 0.14</td>
<td>0.29 ± 0.04</td>
<td>0.03 ± 0.001</td>
</tr>
<tr>
<td>CsA</td>
<td>1.31 ± 0.10a</td>
<td>1.72 ± 0.09a</td>
<td>0.35 ± 0.02a</td>
<td>0.06 ± 0.006a</td>
</tr>
<tr>
<td>CsA+AA</td>
<td>0.76 ± 0.15b</td>
<td>1.32 ± 0.11b</td>
<td>0.25 ± 0.01b</td>
<td>0.04 ± 0.006b</td>
</tr>
<tr>
<td>AA</td>
<td>0.93 ± 0.15c</td>
<td>0.87 ± 0.05c</td>
<td>0.24 ± 0.07c</td>
<td>0.03 ± 0.003c</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D for six animals in each group
One way ANOVA followed by student's t test
Units: Cholesterol, TG, FFA, Phospholipids – mg/g tissue
Values were statistically significant at (P<0.05) for a and b, c- not significant.
Comparisons: aControl Vs CsA, bCsA Vs CsA + AA, cControl Vs AA.
4.11 HAEMATOLOGICAL PARAMETERS

The haematological examinations in the present study revealed abnormal levels of RBC, Hb, PCV, DC and CIC in CsA induced when compared to control rats. A significant decrease in the levels of RBC, Hb, PCV, DC and CIC were observed in CsA induced animals. These changes were reverted to near normal by AA treatment. The erythrocyte membrane contains abundant polyunsaturated fatty acids, which are common targets of oxidative damage by free radical-induced peroxidation. It has been recently reported that the exposure of red blood cells to by-products of lipid peroxidation results in a severe oxidative stress in the cell, which may eventually lead to hemolysis (Tesoriere et al., 2002). Hemolysis may indicate ischemic vascular damage associated with CsA nephrotoxicity, which is the most common and clinically important side effect of CsA (Noel et al., 1992).

Table 9: Hematological parameters in control and experimental group of animals

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Control</th>
<th>CsA</th>
<th>CsA + AA</th>
<th>AA</th>
</tr>
</thead>
</table>

216
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CsA</th>
<th>CsA+AA</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>12.96 ± 0.314</td>
<td>5.46 ± 0.26</td>
<td>7.67 ± 0.30</td>
<td>12.48 ± 0.31</td>
</tr>
<tr>
<td>PCV</td>
<td>38.30 ± 0.748</td>
<td>16.55 ± 1.27</td>
<td>22.33 ± 0.92</td>
<td>36.15 ± 1.378</td>
</tr>
<tr>
<td>CIC</td>
<td>270.65 ± 5.66</td>
<td>121.71 ± 2.76</td>
<td>111.13 ± 2.15</td>
<td>281.14 ± 4.37</td>
</tr>
<tr>
<td>DC</td>
<td>5283.33 ±147.196</td>
<td>4350.00 ±187.08</td>
<td>4800.00 ±178.88</td>
<td>5315.00 ±145.56</td>
</tr>
</tbody>
</table>

**Units:** Hemoglobin content (g/dL), packed cell volume (PCV) (%), Circulating immune complexes (CIC) (OD x 10^3), Differential leukocyte count (DC) (%).

Values are expressed as mean ± SD; n=6

One way ANOVA followed by post hoc test LSD. P<0.05

Comparisons: aControl Vs CsA, bCsA Vs CsA+AA, cControl Vs AA.

### 4.12 HISTOPATHOLOGICAL STUDIES

Figure 39(A) presents the histological changes of renal tissue in control. Histological analysis revealed that Cyclosporine (CsA) administration (Figure B) caused severe and widespread necrosis with tubular epithelial cell degeneration, cast formation in the tubules, interstitial mononuclear cell infiltration of inflammatory cells. CsA induced histopathological renal changes were minimal in animals which received Arjunolic acid (AA) treatment (Figure C). Animals treated with AA alone (Figure D) showed normal renal architecture compared to control (Figure A) group of animals.

The Histopathological study reveals that CsA administration causes morphological changes including glomerular atrophy, blood vessel thickening and hyaline casts in the tubules. Concomitant treatment with NAC (N-acetyl cysteine) attenuated the CsA-induced structural and functional changes in the kidneys. Reactive oxygen species mediate peroxidation of lipid structures of the tissues, resulting in subcellular damage observed by histopathological examination with CsA treatment for 21 days (Can et al., 2004; Nitescu et al.,
Vasoconstriction produced by CsA produces local ischaemia and causes cellular changes like deterioration of membrane integrity and distinct histological changes in the renal structures (Tirkey et al., 2005).

Decrease in structural damage with NAC treatment could be due to the NAC-induced decreased vascular resistance and enhanced tissue perfusion (Boesgaard et al., 1993; Alencar et al., 2003). Improved tissue perfusion with NAC decreased the formation of oxygen free radicals and minimized the CsA-induced cellular damage in renal tissues (Dragger et al., 2004). Sumitra et al., (2001), have clearly portrayed the cardioprotective effects of AA against platelet aggregation, coagulation and myocardial necrosis. Manna et al., (2009) reported that histological studies reveal multiple foci of hemorrhagic necrosis and cloudy swelling of tubules in the kidney of toxin is reduced after treatment with AA.

Figure 39: Effect of Arjunolic acid on histological analysis of kidney in control and Experimental group of animals
4.13 STUDY OF TRANSMISSION ELECTRON MICROSCOPE

The kidney is the target organ for variety of chemicals and drugs due to its peculiar anatomic and physiologic organization. Cell death possesses biochemical or enzymological, functional, immunological and morphological criteria (Kumar, 2007; Kroemer et al., 2009). Due to the rapid expansion in the research on cell death, new putative and correlates modalities of cell death
are apoptosis, necrosis and autophagy (Maiuri et al., 2007; Tasdemir et al., 2008). All these modalities have been clearly described by conventional transmission electron microscopy (TEM). We focused our research on the detection of oxidative damage and modulation by antioxidant AA against renal apoptosis.

The significance of the ability of mitochondria to undergo ultrastructural transformation is not completely understood. The swollen mitochondria might be due to changes in osmolarity that leads to an influx of salts and water into the inner mitochondrial membrane (Oskarsson et al., 1992). The disorientation and fragmentation of the mitochondria cristae might indicate a special affinity for mitochondria membranes, which play a key role in the functional integrity of this organelle (Flower, 1981; Oskarsson et al., 1992). Many experimental studies have demonstrated that apoptosis and necrosis occurred in renal epithelial tubular cells treated by CisPt even if in recent years autophagy has emerged as another mechanism involved in nephrotoxicity (Periyasamy-Thandavan et al., 2008; Yang et al., 2008).

Ultrastructures of control animal mitochondria and with changes in mitochondria during CsA treatment were represented in figure. CsA treated rats causes mitochondrial membrane damage with loss of cristae, shape of the mitochondria is changed, autophagosome filled with cellular debris, lipid
droplets accumulation, lysosomal enzyme deposits with portion of nuclei and cytoplasmic vacuoles (5000x)(500nm, 1µm). CsA induced mitochondrial membrane damages were minimal in animals which received Arjunolic acid (AA) treatment. AA (Figure D) alone treated animals showed normal renal mitochondrial structure with well preserved cristae compared to control (Figure A) group of animals.
Figure 40: Analysis of mitochondrial structure by transmission electron microscopy in the renal tissue

(a) Normal control kidney mitochondria show Microvilli containing proximal tubule, nucleus, half part of nucleus and small amount of mitochondria (2000x). (b) Normal control kidney mitochondria showing normal mitochondrial with preserved cristae structure within membrane foldings near the basement membrane (3000x). (c) Normal control kidney mitochondria possess Basement membrane, basal foldings with mitochondrial cristae (4000x).
Figure 41: Group-II

(a) CsA treated rats shows Autophagosome with disrupted mitochondrial structure and loss of cristae (2000x). (b) CsA treated rats shows Lipid droplets accumulation, mitochondrial structure with loss of cristae, cytoplasmic vacuoles and lysosomal enzyme deposits with portion of nuclei (3000x). (c) CsA treated rats indicate mitochondrial membrane damage with loss of cristae, shape of the mitochondria is changed, autophagosome filled with cellular debris (5000x).
Figure 42: Group-III

(a) CsA + AA treated rats indicates portion of Nucleus, Round and elongated mitochondria filled with cristae and basal foldings (2000x). (b) CsA + AA treated rats shows elongated and round mitochondria with Cristae, membrane foldings near the basement membrane, slightly showing autophagosome (3000x). (c) CsA + AA treated rats shows longer basal foldings with thick basement membrane (5000x).
(a) AA treated rats indicate three tubules, thin basement membrane with basal foldings, many round and elongated mitochondria with well preserved cristae structure (2500x). (b) AA treated rats shows normal structure with basement membrane and basal foldings in mitochondria with well preserved cristae (4000x). (c) AA treated rats indicate elongated and round mitochondria with small portion of nucleus (5000x).
4.14 EFFECT OF ARJUNOLIC ACID ON EXPRESSION OF NFkB

Reactive oxygen species play important role in inflammation; wherein activated cellular infiltrate can produce high levels of both superoxide \((O_2^-)\) and nitric oxide (NO) (Ischiropoulos et al., 1992). Increased levels of \(O_2^-\) (Land et al., 1994) and NO (Nadeau et al., 1995) were reported during chronic rejection of renal allografts and predict a role for oxidative stress during transplant dysfunction. Nitric oxide (NO) plays important role in the regulation of intra-renal vascular tone (Moncada & Higgs, 1993). Its production is mediated by the activity of NO synthase (NOS). Tubular epithelial cells generate NO via iNOS (Mohaupt et al., 1994). The iNOS mRNA was expressed in rat kidney medullary thick ascending limb cells and medullary collecting ducts without any NO inducer (Morrissey et al., 1994). MicroRNAs (miRNAs) are small noncoding RNAs promote the suppression of Mrna translation or lead to mRNA degradation. MiRNAs regulate different cell processes, including apoptosis and anti-apoptosis (Zhang, 2013). Changes in the mitochondrial signaling pathway causes human cancer, cardiovascular and other diseases (Konovalova & Tyynismaa, 2013). Previous studies showed that NO plays important role in the hemodynamic regulation in kidney (Brezis et al., 1991).

Cyclosporine A increases the vasoconstrictor factors endothelin as well as thromboxane in addition to its activation of the rennin angiotensin system (RAS). Also demonstrated is a reduction in the vasodilator factors, prostacyclin, prostaglandin E2, and NO (Textor et al., 1995; Hortelano et al.,
Activation of the RAS by CsA is by two mechanisms, a direct effect on juxtaglomerular cells (JG) and indirectly through arterial vasoconstriction and reduced renal plasma flow. The direct effect of CsA on JG cells was demonstrated in the late 1980s by Kurtz et al., (1988). In this study, primary cell cultures from rat renal cortex containing JG cells showed a threefold increase in renin secretion upon exposure to cyclosporine. Furthermore, no increase in prostaglandin formation or increase in cyclic AMP concentration was observed. This led to the conclusion that CsA stimulated renin secretion by direct effects on JG cells (Kurtz et al., 1988). CsA markedly lowered COX-2 expression which has been shown to have binding sites for NFAT. Therefore, the inhibition of calcineurin by CsA leads to a reduction in NFAT-mediated COX-2 expression and downstream production of arachidonic acid metabolites thereby favoring vasoconstriction (Hocherl et al., 2002). The role of the innate immune system has also been implicated in the nephrotoxicity of CsA.

Recent reports suggest that upregulation of toll-like receptors (TLR) and TNF-α, responsible for dendritic cell maturation, may be stimulated by endogenous, noninfectious ligands (i.e., injured tubular epithelial cells) and stimulates secretion of chemokines that initiate phagocytic influx and immune activation. A study done by Lim et al., (2005) demonstrated through RT-PCR upregulation of TLR2, TLR4, and TNF-α Mrna in CsA-treated rats. They also demonstrated increased levels of MHC-II by immunohistochemistry. Thus, it may be reasonable to conclude that activation of TLR2 and TLR4 by injured
renal tubular cells caused by CsA provides a link between innate immunity and the direct toxic effects of CsA on renal tubular cells (Lim et al., 2005).

NFκB, an important transcription factor is essential for proper function of the immune system. Proliferation of T cells and B cells, activation of macrophages, proliferation and survival of dendritic cells, and activation of T cells are dependent on NFκB activation. Recent evidence showed that NFκB1 mediates inflammatory response and NFκB2 mediates immune response (Shishodia & Aggarwal, 2004). This suggests that suppression of NFκB1 controls inflammation and showed less effect on the immune system. CsA upregulates NF-κB expression and activity (Rafiee et al., 2002; Buffoli et al., 2005; Corsini et al., 2001; Zhang et al., 1999). In unstimulated and uninjured cells, NF-κB is sequestered in the cytoplasm in an inactive state by IkB isoforms, IkB-α being the most abundant isoform. On stimulation, IkB is phosphorylated and degraded, which releases NF-κB and allows it to translocate to the nucleus to act as an active transcription factor. Nuclear translocation of NF-κB in injured endothelial cells were implicated in the transcription dependent expression of cell adhesion molecules and showed the promoter regions of the genes containing NF-κB–binding sites, essential for the expression of these proteins on endothelial cells (Collins et al., 1995).

Recent studies in CIN model, in which free radicals induced oxidative stress, lead to the upregulation of iNOS and NF-κB expression in response to CsA. In their studies, chemical blockade using antioxidants
reduced iNOS and NF-kB overexpression (Buffoli et al., 2005; Magendiramani et al., 2009). CsA increases renal iNOS expression and nuclear translocation of p65 NF-kB subunit. TGF-b is a key molecule in renal disease progression. Beyond its effect on ECM accumulation, TGF-b signaling initiates renal tubular cell pro-apoptotic effectors and EMT in tubular epithelial cells, resulting in tubular degeneration and tubular atrophy. Recent study showed that Myostatin induces mitochondrial metabolic alteration and typical apoptosis in cancer cells (Liu et al., 2013). Previous studies reported that AA effectively ameliorates renal dysfunction by reducing oxidative stress as well as nitrosative stress and deactivated the polyol pathway (Manna et al., 2009).

Figure 44, shows the Western blot expression of NFkB in control and experimental group of animals. In CsA induced (Group 2) rats, increased (Lane 2), expression of NFkB was observed. Treatment with AA (Group 3) showed significant reduction (Lane 3) in the expression of NFkB comparable with control (Lane 1) and Arjunolic acid alone treated groups (Lane 4).
Figure (44): Western blot expression of Nfk-b in control and experimental groups of animals

(A) L1 L2 L3 L4

Nfkβ (65kDa)

(B)

β-actin (42kDa)

Lane 1, Lane 2, Lane 3 and Lane 4 corresponds to control, CsA, CsA + AA and AA respectively. Quantitative data expressing the corresponding protein levels was assessed using Bio-Rad Quantity One ChemDoc software and is expressed in relative intensity arbitrary unit. Values are expressed as mean ± S.D; n=6. One way ANOVA followed by student’s t test using SPSS16 software package. Comparisons: aControl Vs CsA, b CsA Vs CsA+AA, ns-not significant, p<0.05. (Group-I-Control, Group-II-CsA, Group-III-CsA+AA, Group-IV-AA).
4.15 EFFECT OF AA ON EXPRESSION OF BAX, BCL2

The major pathologic feature of chronic cyclosporine nephrotoxicity is progressive TIF with loss of cellularity in areas of fibrosis. Increased kidney-cell apoptosis has been proposed as an important cause of chronic cyclosporine nephrotoxicity (Thomas et al., 1998; Shihab et al., 1999). The Bcl-2 protein protects cells through number of stimuli induced apoptosis like growth-factor deprivation and DNA-damaging drugs. This ability may be related to antioxidant or other cytoprotective properties of the Bcl-2 protein (Hockenbery et al., 1993). The death-repressing activity of Bcl-2 was counteracted by dimerization with Bax. Thus the ratio of Bcl-2 to Bax serves as a rheostat to determine the susceptibility of cells to apoptosis (Hockenbery et al., 1993; Oltvai et al., 1993). Abundant evidence supports the altered level of Bcl-2 and Bax proteins involved in the process of apoptosis in various kidney diseases, including acute kidney failure (Gobe et al., 2000), chronic kidney scarring (Yang et al., 2001), glomerulosclerosis (Wang et al., 2001) and diabetic nephropathy (Ortiz et al., 1997). Cyclosporine treatment resulted in increased Bax expression, whereas the protein expression of the survival factor Bcl-2 was down-regulated. Apoptosis is an essential process in the development and tissue homeostasis of most multicellular organisms; deregulation of apoptosis has been implicated in the pathogenesis of CsA-induced nephropathy (Amore et al., 2000). Caspases which are essential in most types of apoptosis are a 12-member family of specific cysteine proteases (Wolf & Green, 1999).
It has been proposed that the relative ratio of Bax and Bcl-2 determines cell survival following apoptotic stimuli (Oltvai et al., 1993). Recent data suggested that Bax exists in a dynamic equilibrium between the cytosol and mitochondria to control apoptotic priming (Schellenberg et al., 2013). CsA-induced ERK 1/2 and c-JNK phosphorylation in rat kidney. TGF-b1 is capable of activating several other signal transduction pathways in tubular epithelial cells, such as MAPKs (Akoel et al., 2008). TGF-b1 activates the ERK and JNK pathways, and accumulating evidence suggests a function for these signaling pathways in CIN. ERK and PI3K signaling may be involved in CsA-induced reactive oxygen species generation and subsequent renal cell damage (Cantley, 2002). ERK signaling has a critical function in the proliferation of tubular epithelial and myofibroblast-like cells (Masaki et al., 2003). JNK signaling is also involved in TGF-b1-induced fibronectin and CTGF production (Chan & Riches, 2001) and interstitial myofibroblast accumulation (Ma et al., 2007) suggesting a function for JNK in renal fibrogenesis. In progressive renal disease, tubular cell apoptosis precedes manifestations of tubular atrophy, tubular dilatation, and perivascular inflammation (Bottinger & Bitzer, 2002). Yang et al., (2002) also showed that loss of cellularity progresses over time together with the activation of apoptosis-related gene expression until fibrotic tissue replaces the apoptotic cells in a chronic CIN model. Recent data suggested the mechanisms of Cyclosporine-Induced Renal Cell Apoptosis: A systemic review (Xiao et al., 2013).
The intrinsic or mitochondrial pathway of apoptosis contributes to tubular cell injury and death during renal ischemia reperfusion, a major cause of acute kidney injury and renal failure (Castaneda et al., 2003; Devarajan, 2006; Kaushal et al., 2004; Pabla et al., 2009; Padanilam, 2003; Qiao et al., 2005; Saikumar et al., 1998a; Tanaka et al., 2004; Wang et al., 1999; Wei & Dong, 2007). This apoptosis pathway is characterized by the accumulation of Bax in mitochondria, permeabilization of the mitochondrial outer membrane, and consequent release of apoptogenic factors from the intermembrane space (Green & Kroemer, 2004). Recent studies suggested an important role for the regulation of mitochondrial dynamics in mitochondrial injury during apoptosis (Brooks & Dong, 2007; Chan, 2006; Soubannier & McBride, 2009; Suen et al., 2008). CsA/cyclophilin and FK506/FKBP12 complexes can bind to calcineurin, leading to its inhibition (Hara & Snyder, 2007). CsA is well-documented inhibitor of mitochondrial permeability transition (MPT), porous defects in the inner membrane of mitochondria leads to loss of mitochondrial membrane potential (Halestrap, 2009; Lemasters et al., 2009).

Calcineurin (also called protein phosphatase 2B) is a calmodulin-dependent, Ca$_2^+$-activated phosphatase, mediates dephosphorylation of a variety of proteins at serine/threonine sites (Halestrap, 2009). In renal tubular cells, ATP depletion by hypoxia or chemical inhibition of mitochondria leads to increases in intracellular free Ca$_2^+$ (Dong et al., 1998; Kribben et al., 1994; Weinberg et al., 1991), activates calcineurin. Calcineurin dephosphorylates Bad, a proapoptotic Bcl-2 family protein (Wang et al., 1999a). The dephosphorylation causes translocation of Bad from cytosol to mitochondria,
where it interacts with and neutralizes the anti-apoptotic proteins like Bcl-2 and Bcl-XL to facilitate apoptosis (Wang et al., 1999b). The results from the current study suggest that calcineurin may promote apoptosis through yet another mechanism, i.e., Drp1 dephosphorylation and mitochondrial fragmentation. These two mechanisms are not mutually exclusive and may work together to promote mitochondrial outer membrane permeabilization and the release of apoptogenic factors.

Recently reported the downregulation of mitochondrial calcium uniporter by cancer related miR-25 (Marchi et al., 2013). Further investigations should examine the regulation of calcineurin in renal tubular cells and delineate the mechanisms underlying calcineurin mediated mitochondrial damage and apoptosis.

Apoptosis is an active mode of cell death under molecular control, requires energy to proceed (Green & Kroemer, 2005; Riedl & Salvesen, 2007). Mitosis and apoptosis are the basic physiologic processes that regulate cell number. In a healthy organism, 10 billion cells are lost daily through apoptosis and are replaced by mitosis. In the kidney, the rate of apoptosis is particularly intense during development (Koseki et al., 1992). In addition, interventions against Bcl2-like proteins and caspases have used cell-permeable peptides. Among antiapoptotic Bcl2 family proteins, Bcl-2 and BclxL have been most extensively studied in the kidney. Small molecules were used to inhibit apoptosis.
Clinical trials demonstrate the feasibility of the use of caspase inhibition in the clinical setting. Short term use of IDN-6556, an orally active, liver-specific, pan caspase inhibitor, decreases hepatocyte lysis in patients with chronic hepatitis C (Pockros et al., 2007). Its long-term safety remains unclear. IDN-6556 also reduces liver apoptosis in human liver preservation injury and has received an orphan drug label by the US Food and Drug Administration for solid-organ preservation in transplantation (Baskin-Bey et al., 2007). p53 inhibitor pifithrin-α, which prevents apoptosis and protects renal function in ischemia /reperfusion and cisplatin nephrotoxicity, and nanomolecular inhibitors of Apaf-1 (Kelly et al., 2003; Jiang et al., 2006; Vicent et al., 2006). The kidney may be a particularly favorable organ for specific targeting of antiapoptotic molecules. Small molecules may be bound to carriers that lead to specific proximal tubular uptake and organ protection (Prakash et al., 2006). In this study, the ratio of Bax and Bcl-2 was reversed by AA, indicating that AA prevents apoptosis in a CsA-treated rat model. Previous studies reported that AA reduces acetaminophen induced JNK (Jun N-terminal kinase pathway) and downstream Bcl2 and Bcl-xL phosphorylation and prevents mitochondrial permeabilization, loss in mitochondrial membrane potential and cytochrome c release (Ghosh et al., 2009).

Figure 45 & 46 shows the western blot expression of Bax, Bcl2 in control and experimental group of animals. In CsA induced (Group 2) rats, increased expression of Bax and decreased expression of Bcl2 was observed.
Treatment with AA (Group 3) showed significant reduction in Bax and increases Bcl2 expression (Lane 3) comparable with control (Lane 1) and Arjunolic acid treated groups (Lane 2).

**Figure (45): Western blot expression of Bax in control and experimental groups of animals.**

(A) L1 L2 L3 L4

Bax [20kDa]

(B) β-actin [42kDa]

Lane 1, Lane 2, Lane 3 and Lane 4 corresponds to control, CsA, CsA + AA and AA respectively. Quantitative data expressing the corresponding protein levels was assessed using Bio-Rad Quantity One ChemDoc software and is
expressed in relative intensity arbitrary unit. Values are expressed as mean ± S.D; n=6. One way ANOVA followed by student’s t test using SPSS16 software package. Comparisons: aControl Vs CsA, b CsA Vs CsA+AA, ns-not significant, p<0.05. (Group-I-Control, Group-II-CsA, Group-III-CsA+AA, Group-IV-AA).

Figure (46): Western blot expression of Bcl-2 in control and experimental groups of animals.

(A) L1 L2 L3 L4

Bcl-2 (28kDa)

(B)

β-actin (42kDa)

Lane 1, Lane 2, Lane 3 and Lane 4 corresponds to control,CsA,CsA + AA and AA respectively. Quantitative data expressing the corresponding protein levels was assessed using Bio-Rad Quantity One ChemDoc software and is
expressed in relative intensity arbitrary unit. Values are expressed as mean ± S.D; n=6. One way ANOVA followed by student’s t test using SPSS16 software package. Comparisons: aControl Vs CsA, bCsA Vs CsA+AA, ns-not significant, p<0.05. (Group-I-Control, Group-II-CsA, Group-III-CsA+AA, Group-IV-AA).

4.16 EFFECT OF AA ON EXPRESSION OF CASPASE 3

Caspase-3, an important player in the final induction of apoptosis, and a close relationship exists between Bcl-2 expression and caspase activity. The over expression of Bcl-2 inhibits the activation of caspase-3 protease and causes cell death in response to various apoptotic stimuli (Chinnaiyan et al., 1996; Monney et al., 1996; Shimizu et al., 1996). In the kidney, the same mechanism operates in mesangial cells and tubular epithelial cells (Sandau et al., 1997), as well as in an experimental model of progressive TIF (Shihhab et al., 1996). Murine tubular epithelial cell line exposed to cyclosporine underwent apoptosis, with evidence of caspase-3 activation; the administration of caspase inhibitors prevented the cells from undergoing cyclosporine-induced apoptosis (Ortiz et al., 1998).

Caspase-9 is the apical caspase of apoptosis resulting from mitochondrial injury (Sun et al., 1999). Indeed, in renal tubular epithelium, CsA induces Bax aggregation and translocation of Bax to mitochondria, as well as evidence of mitochondrial injury that includes release of cytochrome c and Smac/Diablo and loss of mitochondrial membrane potential. For certain stimuli, like death receptor stimulation or certain chemotherapeutic agents, cytochrome c release from mitochondria may occur secondary to caspase
activation (Robertson et al., 2002; Bossy-Wetzel & Green, 1999). However, in CsA-treated tubular epithelial cells, Bax translocation and cytochrome c release were caspase-independent phenomena, placing them upstream of or parallel to caspase activation. The central hallmark of the mitochondrial apoptotic pathway in vertebrates is the induction of MOMP, the release of cyto-c and the formation of an apoptosome complex (Apaf-1/cyto-c/caspase 9) that directly induces caspase 3 (Tait & Green, 2010). ROS are involved in the oxidation of mitochondrial pores for the release of cyt c (Simon et al., 2000). Recent data suggested that Propofol and magnesium attenuate isoflurane-induced caspase-3 activation via inhibiting mitochondrial permeability transition pore (Zhang et al., 2012). It is also being reported that Cadmium Induces Thymocyte Apoptosis via Caspase-Dependent and Caspase-Independent Pathways (Neelima et al., 2013).

In the mitochondria, Bax promotes the release of cytochrome c to the cytoplasm, where it contributes to the formation of the apoptosome, which leads to the activation of caspase-9 (Sun et al., 1999). Activated caspase-9, in turn, activates caspase-3. That the appearance of active caspase-9 fragments peaked before peak caspase-3 activity and that caspase-9 inhibitor LEHD prevented both the appearance of the caspase-3 active p17 fragment and the development of caspase-3 activity indicates that caspase-9 is activated upstream of caspase-3 in CsA-induced apoptosis. Both caspase-3 and -9 play a vital role in CsA-induced apoptosis. Indeed, inhibition of caspase-9 or caspase-3 activity prevented features of apoptosis and also increased long-term cell survival. The increase in long-term cell survival indicates that
caspase inhibitors rescue both from apoptosis and from other eventual forms of cell death in this model. In this regard, certain forms of apoptotic cell death are not prevented by caspase inhibition; rather, caspase inhibition induces a shift from apoptosis to necrosis (Sane & Bertrand, 1999).

Although increased caspase-3 in this model of renal scarring is novel, gives well-documented role of caspases in the execution phase of apoptosis (Rudel, 1999; Muzio et al., 1996; Zou et al., 1997). Caspase-3 is potentially the most important effector enzyme in apoptosis, providing a common pathway to both death receptor- and mitochondria-dependent apoptotic mechanisms (Rudel, 1999; Cohen, 1997). Caspase-3 is linked to the pathogenesis of other models of renal injury associated with apoptosis. The pivotal role of caspase-3 in the apoptosis machinery makes it an attractive target to regulate apoptosis-related cell death. In vitro, the induction of apoptosis in mouse proximal tubule cells by cisplatin has been inhibited by Ac-Asp-Glu-Val-Asp-H, a known caspase-3 inhibitor (Fukuoka et al., 1998). Apoptosis in hepatic parenchymal cells during endotoxemia (tumor necrosis factor-a mediated) was prevented by injection of Z-VAD, providing a caspase-3 inhibition and effective disease treatment (Jaeschke et al., 1998). It is also been reported that the administration of B-D-FMK (pan caspase inhibitor) was significantly caspase-3 inhibiting and neuroprotective when given by intra cerebral or systemic injection after cerebral hypoxia ischemia (Cheng et al., 1998). More recently reported that Z-VADFMK reduced the caspase-3 activity and prevented the early onset of not only renal apoptosis but also inflammation and tissue injury in a mouse model of renal ischemia.
(Daemen et al., 1999). By these findings, a similar blockade of caspase-3 in progressive renal scarring may provide a novel therapeutic approach to the treatment of renal scarring by controlling inappropriate apoptosis.

In this study, cyclosporine treatment significantly increased, but AA suppressed caspase-3 activity in association with the expression of Bcl-2 protein. Our observations suggest that AA inhibits the Bcl-2 protein–related apoptotic pathway and the subsequent caspase-3 cascade in this model. It is well known that chronic cyclosporine nephrotoxicity is associated with a synthesis of reactive oxygen species and peroxidation (Parra, et al., 1998; Wang & Salahudeen, 1995) and these products may induce kidney-cell apoptosis (Cuttle et al., 2001; Takeda et al., 1999; Lieberthal et al., 1998). Antioxidant therapy such as vitamin E has been shown to prevent cyclosporine-induced toxicity (Parra et al., 1998; Jenkins et al., 2001). AA represents potential therapeutic option to protect renal tissue from the detrimental effects of acute acetaminophen induced nephrotoxicity is a caspase dependent process that involves the activation of caspase-9 and caspase-3 in the absence of cytosolic cytochrome c release (Gosh et al., 2009).

Immunoblot expression of caspase-3 in the control (Lane 1), AA alone (lane 4) and experimental group of animals is represented in (Figure 47). A dark immunoreactive band of Caspase 3 at a position of 17kDa was observed in animals treated with CsA. Caspase 3 expression was found to be increased in CsA induced animals (Lane 2). Upon treatment with AA, the expression of Caspase 3 showed a significant reduction (Lane 3). β-actin was
used as a loading control. Quantitative data expressing the corresponding protein levels was assessed using densitometry and is expressed in relative intensity arbitrary unit.

**Figure (47): Western blot expression of Caspase-3 in control and experimental groups of animals.**

(A) L1 L2 L3 L4

Caspase-3 (17kDa)

(B)

β-actin (42kDa)

Lane 1, Lane 2, Lane 3 and Lane 4 corresponds to control, CsA, CsA + AA and AA respectively. Quantitative data expressing the corresponding protein levels was assessed using Bio-Rad Quantity One Chem Doc software and is expressed in relative intensity arbitrary unit. Values are expressed as mean ±
S.D; n=6. One way ANOVA followed by student’s t test using SPSS16 software package. Comparisons: aControl Vs CsA, b CsA Vs CsA+AA, ns-not significant, p<0.05. (Group-I-Control, Group-II-CsA, Group-III-CsA+AA, Group-IV-AA).

4.17 EFFECT OF ARJUNOLIC ACID ON CYTOCHROME C

Cyclosporine A, a major advance in transplantation, enhancing graft and patient survival; its use, however, is restrained by nephrotoxicity. CsA induces Bax aggregation and translocation to mitochondria, causing permeabilization of the outer mitochondrial membrane, release of cytochrome c and SMAC/DIABLO, and activation of caspase-9 and -3 (Guo et al., 2002; Lassus et al., 2002; Bonzon et al., 2006). Recently reported that PSAP induces a unique Apaf-1 and Smac-dependent mitochondrial apoptotic pathway independent of Bcl-2 family proteins (Ting et al., 2013). Cytochrome c facilitates the oligomerization of Apaf-1 and caspase-9 in the apoptosome, resulting in activation of caspase 9. Caspase-9 activates effector caspases like caspase-3 and -7, resulting in widespread proteolysis and commitment to cell death. Enhancement of cytochrome c release by the PT (permeability transition) is uncertain, outer mitochondrial membrane rupture due to swelling were involved (Vander Heiden et al., 1997), or cytochrome c might be released through unidentified channels dependent on the PT.

Mitochondrial membrane depolarization is a heavily debated issue in apoptosis signaling. Recently reported that p53 opens the Mitochondrial
Permeability Transition Pore to Trigger Necrosis (Angelina et al., 2013). There is agreement on its occurrence, but the causal role for cytochrome c release is clearly disputed (Grimm & Brdiczka, 2007). The permeability transition pore is a polyprotein structure, formed at the site between inner and outer membrane (Marzo et al., 1998a; Marzo et al., 1998b; Crompton et al., 1998; Woodfield et al., 1998; Crompton, 1999). Bax binds to PT pore complex and induces conformational change to open the pore, through which Cytochrome C is released. Since Cytochrome C needs to pass only through the mitochondrial outer membrane. The outer membrane portion of the PT pore seems to consist of not only VDAC (voltage dependent anion channel) but also other molecules, any of which might form a pore large enough for Cytochrome C to pass through. Since Cytochrome C could be released with maintenance of Dc in apoptotic cells (Yang et al., 1997; Kluck et al., 1997; Bossy-Wetzel et al., 1998), there might be another mechanism of apoptosis-associated cytochrome c release, independent of Bax/Bak. Recent study showed that Bcl-2 factor inhibits.

Bax induced cell death while not preventing Bax-induced cytochrome c release (Rosse et al., 1998). It has already reported that AA effectively reduces cytochrome c leakage into cytosol (Ghosh et al., 2009). Many studies confirmed that long-term use of CsA after renal transplantation could induce CCN (Montagnino et al., 2004; Magnasco et al., 2008; Benigni et al., 1999; Takeda et al., 2001; Seron et al., 2001; Marcen et al., 2001).
Therefore CsA-induced renal cell apoptosis might be one of the primary causes in CRD. To reveal the mechanisms of renal cell apoptosis induced by CsA in vitro, analysis of relevant articles and experiments about the mechanisms of renal cell apoptosis induced by CsA and their apoptotic mechanisms are complex. At least four apoptotic pathways mediate renal cell apoptosis in vitro including the Fas/Fas-L, mitochondrial, ER and NO-related apoptotic pathway and synergistically mediated cell apoptosis.

CsA could significantly increase the Fas/Fas-L, FADD and PARP, induce mitochondrial dysfunction and oxidative stress and injure the antioxidant defense system. It also caused Bax migration to the mitochondria, released cytochrome c into cytosol, downregulated anti-apoptotic proteins (Bcl-2 and BclxL) and increased the activity of caspase (Gisslinger et al., 1991; Montagnino et al., 2004; Magnasco et al., 2008; Seron et al., 2001; Marcen et al., 2001; Healy et al., 1998; De Hornedo et al., 2007). They finally activated caspase 9 and 3 which mediate cell apoptosis (Saikumar et al., 1998; Moley & Mueckler, 2000; Boulikas & Vougiouka, 2003; Marchetti et al., 2002). Therefore CsA induces renal cell apoptosis through the Fas/Fas-L pathway as well as mitochondrial pathway with sufficient evidence.

Figure 48 shows the western blot expression of Cytochrome C in control and experimental group of animals. In CsA induced (Group 2) rats, increased expression of Cytochrome C was observed. Treatment with AA
(Group 3) showed significant reduction in Cytochrome C expression (Lane 3) comparable with control (Lane 1) and Arjunolic acid treated groups (Lane 2)

**Figure (48): Western blot expression of Cytochrome-c in control and experimental groups of animals.**

(A) L1  L2  L3  L4

Cytochrome-c  (14kDa)

(B)

β-actin  (42kDa)

Lane 1, Lane 2, Lane 3 and Lane 4 corresponds to control,CsA,CsA + AA and AA respectively. Quantitative data expressing the corresponding protein levels was assessed using Bio-Rad Quantity One ChemDoc software and is expressed in relative intensity arbitrary unit. Values are expressed as mean ±
S.D; n=6. One way ANOVA followed by student’s t test using SPSS16 software package. Comparisons: \(^a\)Control Vs CsA, \(^b\) CsA Vs CsA+AA, ns-not significant, \(p<0.05\). (Group-I-Control, Group-II-CsA, Group-III-CsA+AA, Group-IV-AA).

4.18 EFFECT OF ARJUNOLIC ACID ON IL-6

IL-6 is critical to the inflammatory response to renal injury. IL-6 deficiency diminishes neutrophil accumulation after injury and renders mice relatively resistant to injury. Secondly, neutrophil depletion in wild-type mice significantly reduced HgCl2-induced injury. In this respect, ischemia-induced AKI (acute kidney injury) is similar to both HgCl2- and endotoxemia-induced AKI regarding neutrophil infiltration but dissimilar regarding the contribution of neutrophils to injury (Patel et al., 2005; Singbartl et al., 2005; Kielar et al., 2005; Melnikov et al., 2002). IL-6 expression is an intrinsic element in the cell-mediated inflammatory response that contributes significantly to HgCl2-induced AKI. Lack of IL-6R expression in the kidney precludes an IL-6 – mediated protective response through the classical signaling pathway. Activates STAT3 in the absence of IL-6R expression through the mechanism of IL-6 trans-signaling and increases serum sIL-6R levels after renal injury and causes receptor shedding by infiltrating neutrophils (Jones et al., 2001; Chalaris et al., 2007).

Stimulation of trans-signaling using HIL-6 dramatically reduced renal injury and maintained renal function, demonstrating that IL-6 trans-signaling functions to induce protection in response to renal injury. Multiple factors may participate simultaneously to induce gp130 signaling and activate
STAT3 after renal injury, including IL-11 and leukemia inhibitory factor, which are also induced after renal injury (Lemay et al., 2000; Yoshino et al., 2003). To understand the mechanism by which gp130 activation prevents renal injury, we examined two molecular pathways associated with HgCl2-induced renal injury: Apoptosis and oxidative stress. The amelioration of renal injury through antiapoptotic factors is appealing because it is known that IL-6 and STAT3 upregulate antiapoptotic factors, including Bcl-2 and Bcl-xL (Haga et al., 2003; Kovalovich et al., 2001), which are reportedly upregulated after exposure to HgCl2 (Nath et al., 1996). IL-6 and gp130 signaling is an important physiologic response to renal injury. Renal injury induces local and systemic elevation of IL-6 that promotes neutrophilic infiltration and exacerbates renal injury. The neutrophils can release their membrane-bound IL-6R (Chalaris et al., 2007), via IL-6 trans-signaling, activates STAT3 in the renal epithelial cells. Trans-signaling functions to render protection against oxidative stress and further injury in the surrounding tissue and promotes resolution of injury. This may represent a universal dual role of IL-6 classical and trans-signaling in other types of tissue injury as well. The major pathologic feature of chronic cyclosporine nephrotoxicity is progressive TIF with a loss of cellularity in areas of fibrosis. Increased kidney-cell apoptosis has been proposed as an important cause of chronic cyclosporine nephrotoxicity (Thomas et al., 1998; Shihab et al., 1999). Alterations in the frequency of apoptosis cause disease, characterized by insufficient or excessive number of cells. The loss of function of genes induces apoptosis or the increased expression of genes prevent apoptosis, results in autoimmunity, excess of autoreactive cells (bcl-2, Fas), or neoplasia (bcl-2, p53) (Tsujimoto

Recent data suggest that leukocytes in the inflammatory site are eliminated by apoptosis if there is not an adequate microenvironment of cytokines and cell-preservation factors (Whyte et al., 1993; Mangan & Wahl, 1991; Mangan et al., 1991). The clearance of inflammatory cells by apoptosis might be a mechanisms regulating resolution of glomerular and interstitial inflammation (Savill, 1992) and failure of this clearance may contribute to persistence of the inflammatory process. There is evidence in support of this hypothesis like capacity of mesangial cells to engulf apoptotic neutrophils (Savill et al., 1992) as well as the visualization of apoptotic debris inside mesangial cells during recovery from acute glomerulonephritis (Harrison, 1988). Parveen et al., (2012) showed that TA extract ameliorated the chronic heart failure by reducing oxidative stress to maintain endogenous antioxidant enzymes activities and inflammatory cytokines in rats. Thus, a wide range of beneficial effects of TA have been attributed to its antioxidant properties that made a basis for the treatment of various ailments (Subramaniam et al., 2011; Biswas et al., 2011; Shivananjappa et al., 2012; Parveen et al., 2012).

Figure 49 shows the western blot expression of IL-6 in control and experimental group of animals. In CsA induced (Group 2) rats, increased expression of IL-6 was observed. Treatment with AA (Group 3) showed significant reduction in IL-6 expression (Lane 3) comparable with control (Lane 1) and Arjunolic acid treated groups (Lane 2).
Figure (49): Western blot expression of IL-6 in control and experimental groups of animals.

(A)    L1         L2        L3         L4
IL-6  (21kDa)

(B)
β-actin  (42kDa)

Lane 1, Lane 2, Lane 3 and Lane 4 corresponds to control, CsA, CsA + AA and AA respectively. Quantitative data expressing the corresponding protein levels was assessed using Bio-Rad Quantity One ChemDoc software and is expressed in relative intensity arbitrary unit. Values are expressed as mean ± S.D; n=6. One way ANOVA followed by student’s t test using SPSS16 software package. Comparisons:  aControl Vs CsA,  b CsA Vs CsA+AA, ns-
not significant, p<0.05. (Group-I-Control, Group-II-CsA, Group-III-CsA+AA, Group-IV-AA).

### 4.19 EFFECT OF ARJUNOLIC ACID ON IL-1α AND IL-1β

Cyclosporine reduces production in vitro of several cytokines, required for T cell activation and function. Cyclosporine reduces production of interleukin-1 by splenic macrophages and of interleukin-2 (Bunjes et al., 1981), interferon-γ (Reem et al., 1983) and tumor necrosis factor α and β (Espevik et al., 1987) by T lymphocytes. Interleukin-1 also modulates matrix protein synthesis by fibroblastic cells. In cultured skin fibroblasts interleukin-1 α and β increase collagen type I protein and mRNA (Kahari et al., 1987; Goldring et al., 1987; Postlethwaite et al., 1988) and in cultured gingival fibroblasts Interleukin-1 β increases proteoglycan synthesis (Bartold, 1988). IL-1α remains mostly in the cytosol and associated with the plasma membrane and IL-1β being the cytokine matured by proteolytic enzyme cleavage (Debets et al., 2001). The response of the renal interstitial fibroblast to Interleukin-1 is not known. A hypothesis that remains to be tested is whether cyclosporine alters the cytokine profile of lymphocytes and monocytes in the renal interstitium, resulting in increased matrix protein synthesis by resident stromal cells. Recently reported that JNK1/2 regulates ER-mitochondrial-Ca^{2+} crosstalk during IL-1β mediated cell death in RINm5F and human primary β-cells (Verma et al., 2013).

The role of proinflammatory cytokines, including IL-1, TNF-α, and IL-6, in both physiological and pathological conditions has been a focus of intense investigation. Increasing evidence suggests that secretion of these
products at low levels following tissue injury participate in cellular repair mechanisms. In contrast, when inflammatory cytokines are produced in abundance, pathological sequelae may ensue like dermatotoxicity (Wilmer et al., 1994), atherosclerosis (Munro & Cotran 1988), rheumatoid arthritis, (Brennan, 1994), Chronic liver disease (Tilg et al., 1993). In this respect, over expression of cytokines in the kidney are features of both acute and progressive glomerular disease in humans (Van Goor et al., 1994; Johnson, 1993; Kusner et al., 1991; Ziesche et al., 1994). In particular, TNF-α released from either infiltrating or intrinsic glomerular cells are thought to contribute significantly to glomerular injury in experimental models of toxic or immunologically mediated renal diseases (Baud et al., 1994). Previous studies reported that AA protects both intrinsic and extrinsic pathways against cadmium induced hepatotoxicity (Pal et al., 2011).

Immunoblot expression of IL-1α, IL-1β in the control (Lane 1), AA alone (lane 4) and experimental group of animals is represented in (Figure 50, 51). A dark immunoreactive band of IL-1α, IL-1β at a position of 18 and 35 kDa was observed in animals treated with CsA. IL-1α, IL-1β expression was found to be increased in CsA induced animals (Lane 2). Upon treatment with AA, the expression of IL-1α, IL-1β showed a significant reduction (Lane 3). β-actin was used as a loading control. Quantitative data expressing the corresponding protein levels was assessed using densitometry and is expressed in relative intensity arbitrary unit.
Figure (50): Western blot expression of IL1-α in control and experimental groups of animals.

(A) L1  L2  L3  L4

IL1-α  (18kDa)

(B)

β-actin  (42kDa)

Lane 1, Lane 2, Lane 3 and Lane 4 corresponds to control, CsA, CsA + AA and AA respectively. Quantitative data expressing the corresponding protein levels was assessed using Bio-Rad Quantity One ChemDoc software and is expressed in relative intensity arbitrary unit. Values are expressed as mean ± S.D; n=6. One way ANOVA followed by student’s t test using SPSS16 software package. Comparisons: aControl Vs CsA, b CsA Vs CsA+AA, ns-not significant, p<0.05. (Group-I-Control, Group-II-CsA, Group-III-CsA+AA, Group-IV-AA).
5. INVITRO STUDY

5.1 In vitro assay for Cytotoxicity activity (MTT assay).

Arjunolic acid, a triterpenoid saponin, is a major component present in the isolated extracts of the bark of TA. Triterpenoids, an important class of plant secondary metabolites derived from C_{30} precursors, possess wide range of biological activities, obtained from local market and tested for its protective role against CsA induced functional and structural renal apoptosis.
In the present investigation, the cytotoxicity study of Arjunolic acid was carried out and the results of Arjunolic acid extract at various doses (400, 200, 100, 50, 25, 12.5, 6.25, 3.125 µg/ml) after a week was determined on VERO cells. The cell viability rate of MTT assay was found to be decreased with increased concentration of samples and a plot of concentration versus percent cell viability on the graph produced an approximate linear correlation between them. From the graph, the concentration at which 50% cell viable was determined and the concentration of the extract of Arjunolic acid was found to be 50µg/ml.

In MTT assay, the chloroform methanol extract of Arjunolic acid exhibited significant cytotoxic activity with the concentration value of 50µg/ml. So from this result, extract of Arjunolic acid possesses some cytotoxic effects only at very low concentration. Recent data suggested Diazoxide and cyclosporine A protect primary cholinergic neurons against beta-amyloid (1-42)-induced cytotoxicity (Zeng et al., 2013). Recently reported that antimicrobial, antioxidant and cytotoxic effects of the bark of Terminalia Arjuna have been determined (Shafikur Rahman & Salma Sultana, 2011). It is also been reported that AA protects arsenic induced cytotoxicity in isolated murine hepatocytes (Manna et al., 2007).
Figure 52:

![MTT ASSAY graph]

Table 10: Cytotoxicity effect of Sample on VERO cell line

<table>
<thead>
<tr>
<th>S.No</th>
<th>Concentration (µg/ml)</th>
<th>Dilutions</th>
<th>Absorbance (O.D)</th>
<th>Cell viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>400</td>
<td>Neat</td>
<td>0.14</td>
<td>24.56</td>
</tr>
<tr>
<td>2</td>
<td>200</td>
<td>1:1</td>
<td>0.19</td>
<td>33.33</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>1:2</td>
<td>0.24</td>
<td>42.1</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>1:4</td>
<td>0.29</td>
<td>50.87</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>1:8</td>
<td>0.37</td>
<td>64.91</td>
</tr>
<tr>
<td>6</td>
<td>12.5</td>
<td>1:16</td>
<td>0.45</td>
<td>78.94</td>
</tr>
<tr>
<td>7</td>
<td>6.25</td>
<td>1:32</td>
<td>0.50</td>
<td>87.71</td>
</tr>
<tr>
<td>8</td>
<td>3.125</td>
<td>1:64</td>
<td>0.53</td>
<td>96.49</td>
</tr>
<tr>
<td>9</td>
<td>Cell control</td>
<td>-</td>
<td>0.57</td>
<td>100</td>
</tr>
</tbody>
</table>
Cytotoxicity effect of Sample on VERO cell line

Figure: 53

Normal VERO Cell line

Toxicity- 400µg/ml  Toxicty- 100µg/ml

Toxicity- 50µg/ml  Toxicity- 25µg/ml
REFERENCES (DISCUSSION)


