5. Discussion

The atherogenic diet (AD) was given to rat to elevate the LDL and to create depression in HDL (Nishina et al., 1990; Rajendran et al., 1996). Cholesterol in AD provides a high level of dietary cholesterol and the intestinal absorption of this cholesterol is enhanced many folds by the cholic acid (Joris et al., 1983). High-fat load resulted in increased levels of total cholesterol, triacylglycerols, LDL cholesterol and VLDL cholesterol in the serum (Takeuchi et al., 1999; Zhukova et al., 2014). Results from the serum lipid status of AD fed rats showed increased concentrations of TC, TG, LDL and VLDL, and a decreased concentration of HDL, whereas diosgenin treated rats showed reversal in these results. The high fat diet altered the lipid profile with more low density lipoprotein cholesterol (LDL-C), which is readily oxidized to induce inflammatory mediators in endothelial cells (Matsuzawa et al., 2007; Packard and Libby, 2008).

The hyperlipidemic rat showed focal discontinuity of the endothelium of tunica intima, marked increase in thickness with focal hyalinization in tunica media associated with large number of foam cells (Moushira et al., 2015). Aorta of atherogenic diet induced rat showed subintimal collection of macrophages, foam cells, splitting of tunica media with collection of blood cells and evolving atheromatous plaque formation when compared to diosgenin treated rat. The drug control group showed similar histology like control group. Hence, the analysis of molecular and biochemical parameters in the drug control rat was not performed. Diosgenin dose up to 157 mg/kg.b.wt/day showed no signs of toxicity in experimental animals (Qin et al., 2009; Gong et al., 2011). The diosgenin dosage (80 mg/kg.b.wt/day) selected for the study was lower than the oral no-observed adverse effect level (NOAEL).
The myocardium contains high concentrations of many enzymes, and once the myocardial membrane gets impaired, its contents are released into the extracellular fluid (Akila and Devaraj, 2008; Vijayan et al., 2012). Results showed the increased activities of serum LDH and CK in atherogenic diet induced rat when compared with control and treated group, whereas the LDH and CK showed decreased activities in heart tissue, proving myocardial injury and membrane damage, indicating the progression of arterial damage. Formation of lipid peroxides in biological membrane is a free radical mediated event. The increased level of TBA reactive substances indicates the excessive formation of free radicals and the activation of LPO system, showing damage to the heart in induced group, whereas, treatment with diosgenin showed significant decrease in LPO. Nitric oxide (NO), the crucial modulator of vascular diseases, has a number of intracellular effects that lead to vasorelaxation, platelet adhesion and endothelial regeneration. Damage in the endothelial cell leads to the reduction in activity of endothelial NO synthase with concomitant release of NO. The concentration of NO increased in induced group whereas, in diosgenin treated group it showed a protective effect. Results from the current study also correlate with the report of Vijayan et al., (2012), indicating the progression of arterial damage. Efflux of LDH, indicates cellular damage (Thapa and Walia, 2007) and it is a good stress indicator (Granchi et al., 2010). LDH and CK are indicators of tissue damage (Kristjansson et al., 2016). Elevation of these two enzymes in AD induced rat is the result of increased leakage of these enzymes from tissue into serum. Entrapment of LDL in the sub endothelium, where it is subjected to oxidative modification, involves the process of lipid peroxidation. LPO is an oxidative stress marker (Lee et al., 2012; Strapazzon et al., 2016). Excessive leakage of NO from inflammatory cells results in cellular disturbance leading to heart failure (Barouch et al., 2002; Massion et al., 2003).
Elevated levels of LPO and NO in atherogenic diet induced rat indicate the generation of oxidative stress which led to the cellular disturbance in heart.

Monocytes are the primary inflammatory cell types that infiltrate early atherosclerotic plaques. Their recruitment into plaques drives disease progression (Randolph, 2009). The trans-endothelial migration of leukocytes during the inflammatory process is evoked by the chemokine, monocyte chemotactic protein-1 (MCP-1) (Berliner et al., 1995). MCP-1 plays a key role in the development of fatty streak lesion. Mice lacking receptors for this chemokine are less prone to atherosclerosis (Gerszten et al., 1999; Del Toro et al., 2016). MCP-1 is induced by various stimuli like cytokines, oxidized adducts, and is secreted by endothelial cells, differentiating monocytes and smooth muscle cells. It is expressed mostly in the areas of atherosclerotic lesion formation (Berliner et al., 1995; Boring et al., 1998; Sung et al., 2008). Macrophage differentiation induced by OxyLDL and high fat diet secrete tumor necrosis factor alpha (TNF-α) and its elevation leads to the production of reactive oxygen species (ROS), resulting in endothelial cell dysfunction (Nishida et al., 2008). Blockade of TNF-α improves cardiovascular morbidity and mortality (Zhang et al., 2009; Chan et al., 2016). The studies by Dowlati et al., (2010), Maes et al., (2011), De Kock (2013), Georgia et al., (2015), have shown that patients who endure from hyperlipidemia have elevations in proinflammatory cytokines such as TNF-α and IL-6. TNF-α, designated as pleiotrophic inflammatory mediator, plays a key role in varied pathophysiologic conditions and in linked comorbidities that target more than just the primary tissue (McKellar et al., 2009). Interleukin-6 (IL-6) is significantly upregulated in aortic lesions (Schieffer et al., 2000). Copious IL-6 expression is detected in all stages of atherosclerotic cells such as macrophages, T cells, endothelial cells, and vascular smooth muscle cells (Voloshyna et al., 2014; Wang et al., 2016).
Elevated mRNA expression of MCP-1, TNF-α, IL-6 and NFκBp65 indicated the generation of ROS, endothelial dysfunction and progression of lesion in atherogenic diet induced rat. Suppression in expression of inflammatory mediators was observed in diosgenin treated rat. Generation of ROS also contributes to NFκB activation (Kim et al., 2016) which induced a cascade of inflammatory responses (Akira and Takeda, 2004) in this study. The enhanced production of TNF-α correlated with the accumulation of NFκB in the cytoplasm prior to the stimulus and with its rapid translocation into the nucleus after stimulation (Takashiba et al., 1999). MAC387 (Stoneman et al., 2007) was used as a macrophage differentiation marker to confirm the disease progression which was upregulated in induced group. Immunostaining of MAC387 in aorta indicated the prominent macrophage differentiation in the induced rat when compared to diosgenin treated rat. Immunofluorescence analysis of NFκBp65 in aorta showed the intense nuclear translocation in atherogenic diet induced group. There was a marked reduction of immunoreactivity in the diosgenin treated group.

Notch signaling plays a pivotal role in homeostasis and cardiovascular development. Within blood vessels, Notch signaling integrates with multiple pathways by one of the mechanisms like co-regulation of transcriptional targets (Rostama et al., 2014). Notch stimulation triggers EC inflammatory response by upregulation of adhesion molecules of EC and pro-inflammatory cytokines/chemokines. Upregulation of multiple Notch receptors, including Notch1, Notch3 and Notch4 were noted in atherosclerotic lesions (Liu et al., 2012). Delta-like 4 (DLL4)-induced Notch activation has been observed in macrophages within atherosclerotic plaques (Fung et al., 2007). Dysfunction of ECs was regulated by inhibition of Delta 1 Like (DLL1) homolog, an inhibitor of Notch1 and thereby prevents lesion formation (Schober et al., 2014). Jagged1 is mainly expressed in EC and smooth muscle cells (SMC) and its
overexpression by EC showed a protective effect on EC themselves (Wu et al., 2011; Fazio and Ricciardiello, 2016). In this study, increased mRNA expression of Jagged 1 was observed in induced rats which indicated EC dysfunction. To restitute the homeostasis of EC, the expression of jagged 1 was increased. Hairy and enhancer of split (hes1, 5 and 7) and the Hes-related proteins (hey1 and 2) were significantly higher in atherosclerotic lesions (Liu et al., 2012; Briot et al., 2016). In AD induced rat the interaction of Notch ligand (Jagged1 and DLL1) with Notch receptor (Notch1) was increased, so more NICD was translocated to nucleus, thereby increased the expression of the target genes hes1 and hey1. Immunofluorescence analysis of NICD in aorta also indicated the intense nuclear translocation in the induced group when compared to control and diosgenin treated group.

Differentiation of monocyte into macrophage is the earliest and vital episode for the progressive story of atherosclerosis. Efficiency and molecular mechanisms of diosgenin in inhibition of OxyLDL-induced monocyte-macrophage differentiation was validated through in vitro studies.

Modified LDL provokes a multitude of cellular responses which lead to vascular dysfunction. Lipoproteins have been classified by their electrophoretic mobility (Wasan et al., 2008). In this study, increased electrophoretic mobility of oxidized LDL reflected not only the modification of the lysine residues of apolipoprotein B-100 (Oörni et al., 1997) but also indicated the process of oxidation. Esfandiarei et al., (2011) reported that diosgenin (≥25μM) induced apoptosis in SMC to 60% in a concentration-dependent manner. Different concentrations of diosgenin were used to analyse the viability of THP-1 cells. The viability was more than 90% at the concentration of 14μM. More concern was given to the monocyte to macrophage differentiation and hence 14μM of diosgenin was used as an ideal concentration.
CD36, an 88-kDa transmembrane glycoprotein receptor, is expressed on different types of cells, including monocytes and macrophages (Park, 2014) and is responsible for the uptake of OxyLDL in human macrophages (Rahaman et al., 2006; Xia et al., 2013). Increased levels of CD36 mRNA expression were observed in OxyLDL induced group compared with normal monocyte and diosgenin treated group. Macrophage marker CD68 (Rosenfeld, 2015), also called myeloid specific surface marker (Orekhov et al., 2014), was expressed prominently on OxyLDL induced cells. Inducible enzyme COX-2 which is linked to inflammatory responses (Smith et al., 1996) was expressed prominently in OxyLDL induced and CD68-positive macrophages (Gu et al., 2015). Ample evidence indicates that NFκB is responsible for the transcription of the genes encoding myriad of pro-inflammatory cytokines and chemokines (Lawrence et al., 2005; Hayden and Ghosh, 2011) and it was upregulated in induced cells versus control. The inhibition by diosgenin of the OxyLDL stimulated inflammatory expressions was not attributable to diosgenin cytotoxicity, as assayed by MTT and the expression of the housekeeping genes, β-actin and GAPDH. In order to confirm the monocyte to macrophage differentiation and the linkage between inflammation (caused by macrophage differentiation), immunostaining was done with monocyte marker CD14, macrophage markers CD68, MAC387 and inflammatory marker NFκB. Expression of CD14 was observed in control and diosgenin treated groups and the expressions of CD68 and MAC387 were detected in OxyLDL induced group. The intense and prominent nuclear translocation of NFκB was found in induced versus control cells.

OxyLDL-activated macrophages selectively showed increased Notch 1 expression (Monsalve et al., 2006). Increased expression of Jagged 1 by differentiating macrophages promoted Notch signaling leading to higher translocation of NICD into
nucleus and ultimately expressed more amount of the target gene hes1 (Boulter et al., 2012). In addition to immunofluorescence analysis of NFκB, NICD was also analysed and, like NFκB, it showed intense nuclear expression and translocation in activated macrophages. Diosgenin treatment downregulated the expression of Notch pathway molecules near to that of control cells. The deregulation of Notch signaling has been reported to be involved in various pathological processes (Andersson et al., 2011; Fazio and Ricciardiello, 2016).

Macrophages exhibit phenomenal plasticity and can alter their physiology in response to environmental cues. They are highly multifarious cells that can quickly change their function in response to local microenvironmental signals (Kierdorf and Dionne, 2016). Monocytes induced with OxyLDL, lead to the formation of spindle shaped macrophage and with diosgenin treatment the expectation is that monocyte to macrophage differentiation may be restrained or a half-spindled macrophage morphology, but interestingly the shape was large, round, which is indecipherable in vitro. Changes in morphology of rounded, loosely adherent monocytes to elongated, firmly adherent ones were seen by substituting M-CSF for a mixture of LPS and IFNγ, while with IL-4 stimulation, cells became more rounded and less adhesive (Zajac et al., 2013). They termed elongated and adherent cells as M1 macrophage and more rounded and less adhesive as M2 macrophage. M1 macrophages represent one extreme as pro-inflammatory (Classically activated macrophages) and M2 macrophages represent the anti-inflammatory (Alternatively activated macrophages) (Martino et al., 2002). Then the curiosity was to find out the polarization of macrophage in induced and treated condition.

Cell shape changes have been associated with different functional states of cells (Folkman, 1978), including proliferation and apoptosis (Chen et al., 1997), nuclear
organization (Versaevel et al., 2012), stem cell differentiation (McBeath et al., 2004; Kilian et al., 2010) and muscle cell contractility (Alford et al., 2011). Monocytes and dendritic cells play a central role in pathogen sensing, phagocytosis and antigen presentation. Historically, these cells are also defined by a combination of morphology, physical properties, localization, functions, developmental origins, and expression of a restricted set of surface markers (Villani et al., 2017). Cell shape has an important role in modulating M1 and M2 macrophage polarization. (McWhorte et al., 2013; Zajac et al., 2013). Although it is thought that cytokines and chemokines are the primary regulators of macrophage behavior (Daley, 2005), some recent studies suggest that tissue structure and physical cues in the extracellular environment also contribute to their function (Van Goethem, 2010).

After the notable change in cell morphology, M1 and M2 specific primers were used to confirm the polarization clue. The M1 macrophage-specific CD38, Formyl peptide receptor 2 (Fpr2), G-protein coupled receptor 18 (Gpr18) were expressed in inflammatory condition, whereas the M2 macrophage-specific c-Myc and Early growth response protein 2 (Egr2) (Jablonski et al., 2015) were expressed in diosgenin treated cells. After the confirmation by mRNA expression results, the study was carried out with chemokine protein array and FACS analysis to confirm that diosgenin suppressed the expression of inflammatory mediators, not by preventing the differentiation of monocyte into macrophage, but by allowing macrophage to differentiate into M2, which suppressed the atherosclerotic progression.

The cytokine and chemokine profile of macrophages can define their activation state (Murray et al., 2014). Chemokines are the key mediators of macrophage chemotaxis, but how they differentially regulate M1 and M2 macrophages remains largely unclear. M1 and M2 specific chemokines were analysed using chemokine
antibody array with 38 targets. The chemokines ENA 78, CTACK, Fractalkine, Eotaxin1, Eotaxin 2, GRO, GROα, I-309, I-TAC, IL-8, IP-10, XCL1, MCP-1, NAP2, MIP-1α, MIP-1β, MIP-1δ, MIP-3β, PARC, TARC and RANTES were expressed in OxyLDL induced M1 macrophages. Among these Chemokines, IL-8, ENA 78, Eotaxin1, Eotaxin 2, I-309, IP-10, RANTES, TARC, MIP-3β and MIP-1δ showed significant expression for M1 macrophage. Investigators also attempted to screen chemokines that differentially induce chemotaxis of M1 and M2 macrophages and to explore the underlying mechanism. Among the 41 chemokines that specifically bound to 20 chemokine receptors, CCL19, CCL21, CCL24, CCL25, CXCL8, CXCL10 and XCL2, specifically induced M1 macrophage chemotaxis (Xuan et al., 2015). In the current study, MIP-3β, Eotaxin2, IL-8, IP-10 were expressed in OxyLDL induced macrophages. Elevation of ENA 78 was found in inflamed conditions like rheumatoid arthritis synovial tissue macrophages (Koch et al., 1994), Influenza A virus infected patients (Fu et al., 2016) and in neutrophil extravasation in cardiac ischemia reperfusion injury (Li et al., 2016). IL-8 is produced by various types of cells upon stimulation with inflammatory stimuli (Harada et al., 1994) and inflammasome activation (Gavrilin et al., 2009; Bobryshev et al., 2016). Expressions of Eotaxin 1 and Eotaxin 2 were significantly increased in eosinophilic myocarditis (Diny et al., 2016). MIG and Eotaxin3 were upregulated in sclerotic calcification of the aortic valve (Anger et al., 2007). In contrast, expression of Eotaxin3 was observed in control monocytes in this study. MCP-1, MIG and IP-10 are the inflammatory chemokines that played a key role in cerebrospinal fluid after subarachnoid hemorrhage (Niwa et al., 2016) and MIG, IL-8 and MCP-1 were used as M1 markers (Goparaju et al., 2015). RANTES and MIP-1α and MIP-1β are crucial mediators of inflammation (Rot et al., 1992), recruitment of infiltrating inflammatory cells and disease severity (Miyagishi et al.,
RANTES was also elevated by peritoneal fluid macrophages in endometriotic lesions (Lebovic et al., 2004). All the aforementioned chemokines induced the expression of M1 macrophage which represents the inflamed condition. The current result also paralleled the M1 macrophage differentiation which mimics inflammation.

M2 macrophages are often termed as wound-healing macrophages (Forbes and Rosenthal, 2014; Rőszer, 2015). Diosgenin drove OxyLDL induced monocyte to M2 macrophage differentiation instead of M1, which was evident by the expression of the M2 specific chemokines MDC, BLC, MIP-1α, TECK, SDF-1α and SDF-1β. The macrophage-derived chemokine (MDC) is expressed by M2 macrophage (Qian et al., 2010; Sun et al., 2016) and not expressed in stressed condition (Zeyda et al., 2010). B lymphocyte chemokine (BLC) was significantly increased in diosgenin treatment which coincides with multiple sclerosis treatment by random amino acid copolymer poly (Y,E,A,K)(n) (Copaxone®) and a second generation copolymer poly (Y,F,A,K)(n) (Kovalchin et al., 2011). TECK and TARC showed expression of M2-associated chemokines (Mantovani et al., 2004) and tolerant monocytes acquire an alternative-activation program, with high expression of M2 immunomodulatory genes TECK (Porta et al., 2009). Stromal cell-derived factor-1, SDF-1α and SDF-1β modify macrophage differentiation towards healing. Majority of chemokines are expressed in pro-atherogenic condition whereas the SDF is expressed in anti-atherogenic condition (Abi-Younes et al., 2000). Disruption of this chemokine leads to lesion formation and plays a key role in atherogenesis in mice (Zernecke et al., 2008). Cytokines associated with M1 activation may be produced by M2 macrophages also, such as IL-6, TNF-α (Sica and Mantovani, 2012; Murray et al., 2014) which was observed and explained earlier in this current study. All the aforementioned chemokines induced the expression of M2 macrophage which represents the diosgenin treated condition.
Summarising the above results, the OxyLDL cues M1 macrophage polarization, whereas diosgenin stimulates M2 macrophage polarization. An interesting finding was that, whereas a number of chemokines were found to induce specifically M1 and M2 macrophage expression, MIP-1α (CCL3) was the only chemokine identified to be expressed in both M1 and M2 macrophages. Apart from M1 and M2 specific chemokines, monocyte specific chemokines were also analysed. Eotaxin 3, GCP2 and HCC4 showed expression for monocytes which was the control group. Among the 32 chemokines, CTACK, Fractalkine, GROα, I-Tac, XCL1, MCP1, NAP2 were a few expressed in both control and induced condition. TARC, CCL23 and MPIF1 is the unique chemokine which was expressed by control, induced and treated cells. CCL28, CXCL16, MCP2, MCP3 and MIP 3α were specific chemokines which showed no expression in control and experimental cells.

Flow cytometry analysis revealed that OxyLDL induced group showed high expression for M1 macrophages, whereas diosgenin treated group expressed high levels of M2 macrophages and low M1 macrophages. Control group with THP-1 cells showed no expression for both the M1 and M2 macrophage markers.

M1 macrophage polarization was observed in OxyLDL induced monocytes, which are pro-inflammatory in nature, whereas M2 macrophage polarization was noticed in diosgenin treated monocytes, which exhibits anti-inflammatory properties. Further, the result was validated by immunostaining of M1 and M2 markers in aorta of atherogenic diet induced and diosgenin treated rat. CD64 and CD23 were classified as M1 and M2 markers respectively (Martinez et al., 2014). Expression of M1 macrophage was noticed in AD diet induced aorta and M2 macrophage was observed in diosgenin treated aorta. Specific changes in cell morphology of M1 and M2 were not noticed in aorta as they were seen in in vitro study. This is the first report that, diosgenin
showed its anti-inflammatory property by the unifying mechanism of modulating macrophage to M2 polarization which suppressed the inflammatory mediators.

Concluding that diosgenin treatment led to M2 macrophage expression in both in vitro and in vivo model systems, another interesting finding was that the two transcription factors NFκB and NICD were upregulated in atherogenic diet-induced rats and the same was confirmed in vitro using THP-1 monocyte cells exposed to OxyLDL to induce macrophage differentiation. As a next step to confirm the regulation of NICD and NFκB, the NICD inhibitor DAPT (Geling et al., 2002; Liao et al., 2016) was used to check the expression of NFκB and the NFκB inhibitor dexamethasone (Aghai et al., 2006) was used to examine the change in expression level of NICD. Macrophage marker MAC387 was used to confirm the macrophage differentiation. The inhibitors were added on the 4th day of the experiment and the differentiation of macrophage was confirmed with co-immunostaining of CD14 and MAC387.

A synthetic glucocorticoid, dexamethasone (DEX), showed 20-30-fold higher immunosuppressive potency compared to cortisone (Kinne et al., 2000; Haim et al., 2014). The current study goes along with Inoue and Tanabe (1998) who reported the induction of NFκB by lipopolysaccharide and suppression by DEX in U937 cells. In DEX treated cells, NFκB was gradually inhibited and the concomitant suppression of NICD was observed which showed the inhibition of NFκB activation downregulated NICD. Exposure of different sources of macrophage to DEX resulted in a wide range of responses, including apoptosis of macrophages in the nervous system (Nguyen et al., 1997). Contradictorily, it enhanced the viability of murine RAW 264.7 macrophages (Fong et al., 2007) and blood monocyte-derived macrophages (Barczyk et al., 2010). The level of expression of MAC387 did not show significant changes with DEX.
treatment. Inhibition of NFκB subsequently downregulated the expression of NICD, but it neither enhanced nor diminished the viability of the differentiated macrophage.

A potent and specific inhibitor of γ-secretase enzyme, N-[N-(3, 5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT), was used to block the proteolytic processing and reduce the release of NICD (Arumugam et al., 2006; Li et al., 2009). Differentiating macrophage treated with different concentrations of DAPT showed gradual decrease in the expression of NICD. Even though the expression of NICD was declined placidly, the expression of NFκB was not altered, but simultaneous downregulation of macrophage marker MAC387 was detected versus OxyLDL induced control. The results of this study suggested that NFκB might interact with NICD in differentiating macrophage, but, NFκB was not dependent on NICD. As Notch signaling is mainly involved in cell fate decision, inhibition of NICD affects the differentiation of macrophage. Data showed that NICD enhanced NFκB nuclear translocation and DNA binding in LPS-activated macrophages (Monsalve et al., 2006) but whether the NICD inhibition affected NFκB expression in differentiating macrophage on exposure to OxyLDL is not clear. These discrepancies could be related to the differentiation state of the cells, the type of cell/inducer, or even to functional differences among the different Notch receptors and their interaction with NFκB.

Both DEX and DAPT were added to the differentiating macrophage to ascertain the NFκB and NICD expression. NFκB, NICD and MAC387 expression were unvaryingly reduced. Independent and combined nuclear expression of NFκB and NICD were observed in OxyLDL induced cells which was shown by co-immunostaining of NFκB and NICD. Flow cytometry analysis revealed that control group with THP-1 cells alone expressed high levels of CD14 and the OxyLDL induced cells expressed high levels of MAC387 and low CD14. NFκB inhibited cells at 1μM
and 16µM showed no significant changes in the expression level of MAC387, whereas NICD inhibited cells showed significant suppression on macrophage differentiation at 16µM concentration when compared to 1µM concentration. This is first report that, NFκB may regulate NICD in respect to inflammation but not influencing macrophage differentiation, whereas, NICD independently influenced macrophage differentiation but not regulating NFκB expression. It is also possible that both NFκB and NICD might bind to independent promoter sites and cooperate to enhance transcriptional activity (Monsalve et al., 2006).

In this study, macrophage differentiation which induced the formation of inflammatory mediator was not significantly suppressed by the inhibition of NFκB using Dexamethasone. But the diosgenin modulates monocyte to M2 macrophage and thereby suppressed the expression of NFκB, which in turn reduced the inflammatory reaction.

Antibiotics are consumed for common causes like strep throat, food poisoning, sinus, ear pain and plaque infections including tooth ache which are caused by bacteria. Cold, flu, cough and bronchitis are some of the common illnesses caused by viruses, for which, medicating with antibiotics is of no use. But, we have developed a tendency to take antibiotics just like chocolate. India is the largest consumer of antibiotics (Van Boeckel et al., 2014). Antibiotics have led to an extraordinary decrease in morbidity and mortality associated with bacterial infections. Yet, despite the great benefits, antibiotic use has been linked to various adverse side effects, including ototoxicity (Brummett and Fox, 1989), nephrotoxicity (Mingeot-Leclercq and Tulkens, 1999), tendinopathy (Khaliq and Zhanel, 2003), mitochondrial dysfunction and oxidative damage (Kalghatgi et al., 2013). Cardiovascular disease holds a major part as a cause of death at all ages in India (Jha and Laxminarayan, 2009) and at the same time
outpatient purchases of antibiotics from retail outlets in India peaked in crores (IMS personal communication, 2009). Ciprofloxacin (a fluoroquinolone) is available in all primary, secondary and tertiary care centers (Kotwani and Holloway, 2013). In addition, antibiotics are widely used as growth promoters in poultry. According to the national policy of containment of antibiotic resistance, India, 2011 released by the Directorate General of health services, New Delhi, there are no regulatory provisions regarding the usage of antibiotics in poultry in the Indian scenario and the fluoroquinolone in poultry led to drug resistance in human.

Ciprofloxacin, a common bactericidal antibiotic induced intercellular ROS generation and led to DNA, protein and lipid damage in various human cell lines (Kalghatgi et al., 2013). To establish a link between atherosclerosis and antibiotic usage and as mentioned earlier, monocyte to macrophage differentiation is the initiation phase of the atherosclerosis, monocytes were supplemented with ciprofloxacin and later induced with OxyLDL to analyse the monocyte differentiation.

Cell viability assay was performed in THP-1 cells with different doses of Ciprofloxacin. The viability was more than 85% at the concentration of 5μg/ml. More concern was given to the monocyte differentiation on addition of OxyLDL and hence 5μg/ml of Ciprofloxacin was used as an ideal concentration. Clinically relevant dose of Ciprofloxacin at 10μg/ml was used in human mammary epithelial cells and various human cell lines to induce ROS formation (Kalghatgi et al., 2013). Yet, the concentration used in this study was less than the clinically relevant dose.

Major classes of bactericidal antibiotics are demonstrated to induce oxidative damage in bacteria, leading to the generation of ROS (Kohanski et al., 2007; Foti et al., 2012). That, cell death by bactericidal antibiotics does not depend on the ROS generated is currently a matter of debate (Keren et al., 2013; Liu and Imlay, 2013). However, the
present study focused on monocyte differentiation only. Reactive oxygen species and oxidative damage are thought to play an important role in many human diseases including cancer, atherosclerosis, other neurodegenerative diseases and diabetes (Eruslanov and Kusmartsev, 2010). H$_2$DCFDA (2’,7’-dichlorodihydrofluorescein diacetate) is non-fluorescent but in the presence of ROS, when this reagent is oxidized, it shows green fluorescence. Acridine orange can penetrate normal and early apoptotic cells with intact membranes, fluorescing green when bound to DNA. Ethidium bromide can only enter cells with damaged membranes, such as late apoptotic and dead cells, emitting orange-red fluorescence when bound to concentrated DNA fragments or apoptotic bodies (Ribble et al., 2005). ROS and apoptosis were measured in time and dose dependent manner using immunofluorescence analysis. ROS was generated in all the experimental groups with a single dose of Ciprofloxacin at 5μg/ml and the maximum fluorescence was observed after 12h, 24h, 48h, 72h and declined after 96h and 120h. Cell death was prominently observed at 120h in group II. In group III, addition of Ciprofloxacin to monocytes at every 24h upto 120h showed the maximum ROS generation in 24h, 48h and 72h and after that there was a decline in ROS production. Apoptosis, it started at 48h, gradually increased and was more prominent in 96h and 120h of Ciprofloxacin supplementation. Emission of orange fluorescence at 72h and 120h of Ciprofloxacin inclusion to monocytes suggested the DNA fragmentation and the presence of apoptotic bodies.

After the measurement of ROS and cell death, cell morphology was studied by supplementing Ciprofloxacin with a dosage of 5μg/ml at every 24h upto 120h and OxyLDL was added to the experimental groups. Indecipherable cell morphological changes were observed in cells supplemented with two doses of Ciprofloxacin and OxyLDL was added at 48h and the change in cell morphology was observed upto 14
days (group III cells). Foam cell formation was observed and was confirmed by oil red O staining. In addition, \textit{in vitro} plaque formation was also observed in Ciprofloxacin added group. Further, the protein expression pattern was analysed with control and Ciprofloxacin supplemented group. SDS-PAGE showed the appearance of a single protein in ciprofloxacin added group. The band was excised and analysed with MALDI-TOF. The spectral mass/charge (m/z) value was blasted in Mascot database search-matrix science. Unfortunately, significant hit of a protein was not obtained. Then the protein sample was subjected to LC-MS/MS study and the spectral m/z value was again blasted in Mascot database search-matrix science and a significant hit of protein sequence with 98 score was obtained. The protein was chain A, human serum albumin complexed with Myristate with a molecular weight of 68 kDa. Mascot score above 68 is considered as significant and significant hits showed the most common myristic acid association with serum albumin. Several proteins which undergo myristoylation in eukaryotic cells have already been reported (Carr \textit{et al.}, 1982; Poli \textit{et al.}, 1991). Earlier interesting observations by Aderem \textit{et al.}, (1986; 1988a, b) with mouse macrophages had restricted their analysis to a limited number of proteins containing covalently attached myristic acid. A key mechanism whereby macrophages induce inflammation is through the secretion of fatty acids like arachidonic acid (20:4). Treatment of cells with bacterial lipopolysaccharide (LPS) increased the maximal amount of arachidonic acid release induced by zymosan (Aderem \textit{et al.},1986). Post-translational modifications (PTMs) are identified specifically in cardiac proteasomes (Kimura \textit{et al.}, 2012). Proteasome subunits are subjected to several PTMs, including N-terminal acetylation, lysine acetylation, methylation, phosphorylation, N-myristoylation, O-linked glycosylation, S-glutathionylation, ubiquitination (Cui \textit{et al.}, 2014). Bacterial lipopolysaccharides (LPS), phorbol myristate acetate (PMA) and zymosan induce the
myristoylation of specific macrophage proteins (Aderem et al., 1986). In cells induced with bacterial lipopolysaccharides, fatty acid is incorporated into proteins with molecular mass of 68 kDa. Myristoylation of the 42 and 68 kDa proteins is involved in the priming signal, it is further possible that the covalent transfer of the myristoyl moiety of LPS to macrophage proteins might be the molecular basis of priming (Aderem et al., 1986). Myristic acid is also known to maintain hypercholesterolemia along with increased levels of LDL which are the two key factors involved in progression of atherosclerosis (Zock et al., 1994; Speziali et al., 2016). This is first report that, myristic acid was incorporated into a protein with 68 kDa molecular mass in supplementing monocytes with ciprofloxacin and OxyLDL, which could be a reason for the observed foam cells and in vitro plaque formation, as myristic acid primes the signal for atherogenesis. Flow cytometry analysis revealed that Ciprofloxacin supplemented cells (THP-1 cells added with Ciprofloxacin at 5µg/mL at every 24h for 2 days, then induced with OxyLDL) showed higher expression of M1 macrophages than OxyLDL induced cells. Control group with THP-1 cells showed no expression for both the M1 and M2 macrophage markers.

Further, to confirm whether taking ciprofloxacin fosters atherosclerosis, protein from three groups including monocyte, M1 macrophage and monocyte with addition of Ciprofloxacin for 2 days and induced with OxyLDL were analysed for chemokine expression of 32 targets in an array.

The chemokines CCL23, CTACK, Eotaxin 3, GCP2, GRO-α, BCC4, I-TAC, NAP2 and SDF-1α were expressed by monocytes in which, CTACK, Eotaxin 3, GCP2, GRO-α, HCC4 and MPIF1 showed significance for monocytic expression. ENA 78, CXCL16, Eotaxin 1, Eotaxin 2, Fractalkine, IP-10, MIG, MIP-3β, SDF-1β and TECK showed significant expression for ciprofloxacin induced cells. These chemokines were
expressed in inflammatory condition as described earlier in the M1 macrophage study. Chemokines IP-10 and MIG are considered as key human markers which are expressed by M1 macrophages as proinflammatory mediators (Chinetti-Gbaguidi et al., 2015) was also showed significant expression in Ciprofloxacin added cells. In particular, the high expression of CXCL16 and Fractalkine were observed particularly in ciprofloxacin induced group. M2 specific chemokine TECK was also expressed in ciprofloxacin induced cells. Fractalkine is an unusual membrane-bound chemokine that mediates functions including migration, adhesion, and proliferation chemotaxis through the CX3CR1 receptor. Recently, functional polymorphisms in the human CX3CR1 gene have been observed to be associated with coronary artery disease (Lucas et al., 2003; White et al., 2012; Polyák et al., 2016). CXCL16 plays a pro-inflammatory role in human atherosclerosis (Lehrke et al., 2007) which was expressed by ciprofloxacin induced cells which is evidence for the role of inflammation.

Ciprofloxacin supplemented for 2 days, then induced with OxyLDL showed high intensity of the ROS generation and the results of apoptosis staining showed initiation of DNA fragmentation. Indecipherable cell morphologies were observed which include foam cell and in vitro plaque. Flow cytometry data showed that a high percentage of Ciprofloxacin added cells expressed M1 macrophages which possess pro-inflammatory properties. The chemokine antibody array also revealed the expression of M1 specific chemokines. Protein profiling and LC-MS/MS data showed incorporation of myristate, which primes atherogenesis. All together it was shown that improper use of antibiotics can be disastrous.