Chapter 4:

Results and Discussions

4.1. DETERMINATION OF TOXICOLOGICAL PARAMETERS

4.1.1. Acute toxicity test

During the experimental trials, no animal died in the acute toxicity test. The animals were observed and monitored regularly at an interval of 2 hours on the experiment day. The animals were observed at least thrice in the successive seven days. All the animals were alive for the next 7 days and no death or change in the physiology and/or behavior was observed (**Table 4.1**). Normal gain in weight was observed in all the animal groups and no gross abnormal findings were documented in any of the groups.

Table 4.1: Behavioral changes in the rats following acute toxicity test observed in a period of 24 hours after feeding with *Aloe* leaf extract.

Groups	**PC**	**TA1**	**TA2**
Number of Animals	6	6	6
Number of Deaths	None	None	None
Abnormal motor activity	-	-	-
Salivation	-	-	-
Fur irritation	-	-	-
Sleep/ dizziness	-	-	-
Lethargy	-	-	-
Diarrhea	-	-	-

PC denotes positive control; TA1 and TA2 refer to experimental groups receiving doses at 2 and 5 g of *Aloe* crude gel/kg b.w respectively of model animals. "-" denotes absence

In 1981, OECD incorporated acute toxicity test as well as LD_{50} test into its new guideline. It was further modified in 1987 and subsequent years. This was known as the 'limit test' where upper dose limit value was set at 5g/kg b.w.; the substances are suspected to be non-toxic if there is no sign of toxicity of the drug like compound at the upper dose limit level. In this regard, the precise LD_{50} value determination is not necessary and further acute toxicity testing is not required (Erhirhie *et al.*, 2018). Different plant extracts and herbal remedies have shown their non-toxic properties in the limit test. Java tea leaf extracts (*Orthosiphon stamineus*) in aqueous, aquo-ethanolic and ethanolic solvents showed no toxic properties upto upper dose limit (Pariyani *et al.*, 2015). Similarly, *Syzygium guineense* leaves methanolic extract showed no toxic properties when acute toxicity tests were performed (Loha *et al.*, 2019). However, experimentations on the acute toxic effect of crude unprocessed freshly harvested *Aloe vera* gel were not done in detail prior to the present work.

The acute toxicity test (**Table 4.1**) results indicated that the unprocessed, orally-fed *Aloe vera* gel in Wistar albino rats was not toxic when the rats were fed with a single large dose of 5 g/kg b.w. (further validated by a 7-day long observation), which also briefly indicated that any further testing for unprocessed *Aloe vera* gel for acute toxicity was not necessary (Roopashree *et al*, 2009). The *Aloe* gel was therefore considered to be non-toxic up to the dose of 5 g/kg b. w. and the lethality value was considered "unclassified" up to the feeding range.

4.1.2. Sub-chronic toxicity test

We observed that out of 6 animals per group, only one animal died in the T2 group (i.e. the medium-dose group (2 g/kg b.w.). A post-mortem anatomical investigation was performed on the dead animal, and it was diagnosed with numerous cysts in the liver. Therefore, the death can be considered as unrelated to the toxic side-effects of the *Aloe vera* gel, as no other toxicological symptoms were observed in the other living animals of the same or other dose groups. Body weight of the experimental animals also showed no significant difference among all the dose groups when measured against the untreated animals (**Fig. 4.1**). The changes observed in the histological sections of the liver and kidney tissues were also non-significant (**Fig. 4.2**). The different enzymatic parameters of the serum, like triglycerides, cholesterols, ALP, creatinine, HDL-D, LDL-D, SGPT, SGOT (**Fig. 4.3; A, B, C, D, E, F, G** and **H** respectively) showed no major significant differences between the experimental groups and the control animal group. In addition, no change in the blood sugar level in the experimental groups was observed. The triglyceride level was normal in T1 and T3 but slightly decreased in T2. The serum cholesterol concentration was decreased in T2 and T3, while the concentrations of serum creatinine increased in T2.

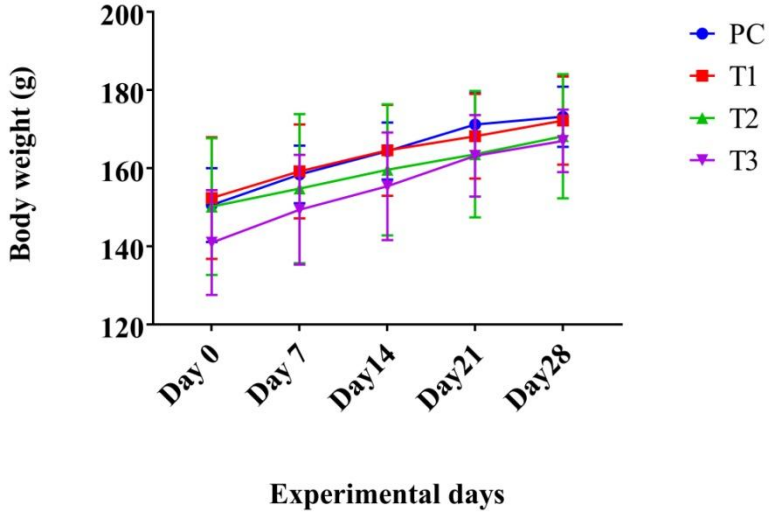


Figure 4.1. Diagram showing the increment in mean body-weight in different groups of experimental rats of sub-chronic toxicity test. PC refers to positive control animals, T1, T2 and T3 refer to the *Aloe vera* gel homogenate-treated groups, with a single daily-dose schedule at doses of 1, 2 and 4 g/kg b.w. respectively. No significant difference in the degree of weight-increment was seen among the groups.

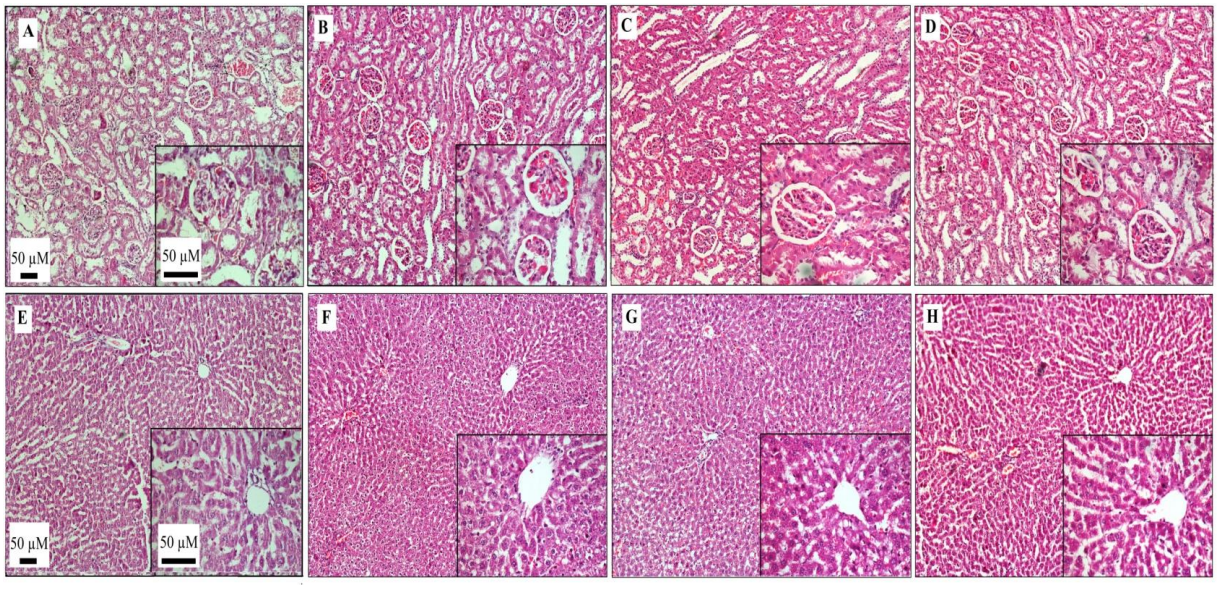


Figure 4.2. Histological study of Kidney (in the upper panel) and liver (in the lower panel) of experimental rats of sub-chronic toxicity test. A and E represent PC group, B and F represents T1, C and G represents T2 and D and H represents T3. The insets of each picture show 40X magnification where as the main picture shows 10X magnification. The size of the bar is in µM. PC refers to positive control animals, T1, T2 and T3 refer to the *Aloe vera* gel homogenate-treated groups, with a single daily-dose schedule at doses of 1, 2 and 4 g/kg b.w. respectively.

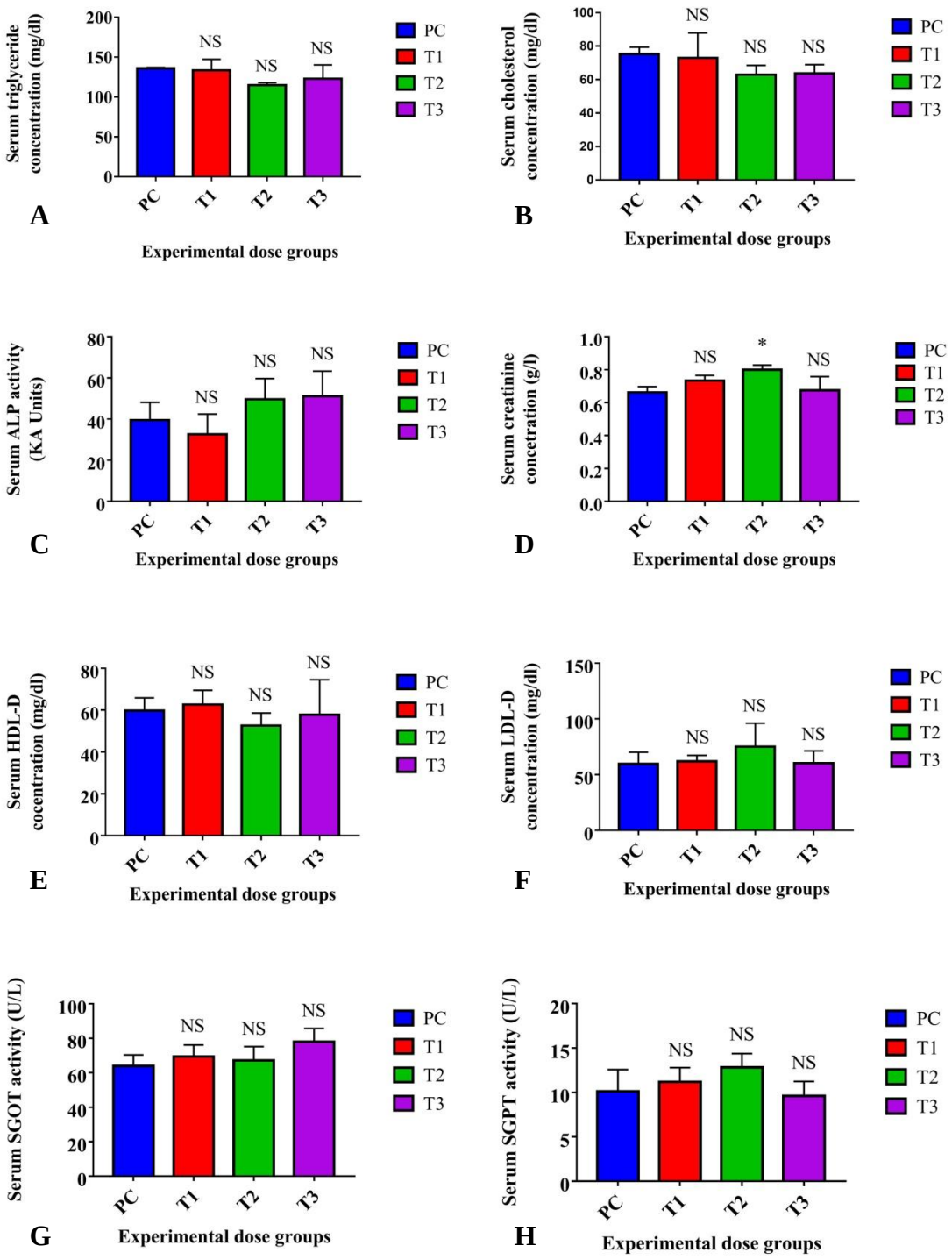


Figure 4.3. Different toxicological parameters measured in sub-chronic toxicity test experimental rats, fed with *Aloe vera* gel homogenate. * denotes significance at P≤0.05. PC refers to positive control animals; T1, T2 and T3 refer to the *Aloe vera* gel homogenate-treated groups, with a single daily-dose schedule at doses of 1, 2 and 4 g/kg b.w. respectively. NS denotes non-significant variation with PC. NS = non-significant; * = P< 0.05; ** = P < 0.01; *** = P< 0.001

The present study has explored in detail, the sub-acute toxic effects of the crude unprocessed parenchymal gel obtained from wild *Aloe vera* harvested from the sub-Himalayan part of West Bengal. For sub-chronic toxicological tests, a loss in the body weight is an indicator of immediate toxic effect of a product (Brautbar and Williams, 2002) and no such change in body weight was observed among the animals of different groups consuming different *Aloe* gel doses during the 28-day long gel feeding schedule. No change in the feeding behavior, in the circadian clock, and in water or food intake was observed in the experimental animals during the study. Our findings indicated that there is no detectable harmful interaction between the major physiological attributes of the model animal system and *Aloe vera* gel constituents when the gel is orally consumed. Histological analysis of kidney and liver tissues supported the results obtained from the analysis of serum enzymes. There were no striking alterations in the structures of the stained sections of kidney and liver between the experimental groups and PC. Kidneys showed well-formed Bowman's capsule and well visible proximal convoluted tubules around the highly vascularized glomerular region. In the liver histological sections, the cells forming the liver showed no deformities. In our study, in all the experimental groups, both organs showed normal structure and arrangement of cellular components. (**Fig. 4.2**). This was further supported by the comparison of serum biochemical (**Fig. 4.3**) parameters of *Aloe* gel-treated groups and control group animals. Blood glucose and hemoglobin estimates were normal in all experimental groups (data not shown).

The serum creatinine was slightly elevated in the T2 group (**Fig. 4.3 D**), which could indicate kidney malfunction but it was not supported by the histology of the kidney of the T2 group animals when compared with those of the other groups (**Fig. 4.2**). Moreover, the other experimental groups receiving different *Aloe* gel doses showed no such changes in the levels of serum creatinine. As a result, an obvious reason for the change of one serum constituent level (creatinine) in one experimental group (i. e. T2) could not be specified.

Cholesterol, which is a 'bad fat', decreased non-significantly in the T2 and T3 groups (**Fig. 4.3, B**), indicating that *Aloe vera* gel consumption reduced the cholesterol content of the serum, at least at a high dose. However, all groups had normal triglyceride content of 100-140 mg/dL (**Fig. 4.3 A**). LDL-D and HDL-D (**Fig. 4.3, E** and **F**) values showed no

significant changes in the experimental groups compared to PC. ALP, secreted from the bile duct and liver, is a bile duct malfunction marker when SGOT and SGPT levels are normal; on the other hand, SGPT, SGOT and ALP together constitute the array of markers for liver damage (Giboney, 2005; Kew 2011). ALP, SGPT and SGOT remained within normal range at the end of the experimental feeding schedule (**Fig. 4.3, C, G** and **H** respectively). In addition to this, any damage in liver parenchymal cells results in an elevation of SGOP and SGPT levels (Giboney 2005), which was assessed to be absent in this study. The obtained normal values of serum enzymes are strongly supported by the normal appearance of the organs in histology.

Sub-chronic toxicity tests following the OECD guidelines provide detailed idea regarding the physiological and biometric changes in the body during a moderate-long (28 days) exposure to the drug or drug-like compounds. Almost all the herbal medicines and plant extracts are screened through the process to ensure a safe consumption and lack of side-effectivity. Extract of *Syzygium guineense* (Loha *et al.*, 2019) showed no toxic effect at a sub-chronic dose of 1500mg/kg b.w. Some recent studies include the ethanol extract of *Pericampylus glaucus* (Kifayatullah *et al.*, 2015), *Cassia fistula* (Abid and Mahmood, 2019), *Withania frutescens* (Moussaoui *et al.*, 2020), and even some species of genus Aloe except *Aloe vera* found in Ethiopia. All these plant extracts have been concluded to have a non-toxic effect at sub-acute level at higher doses. The serum biochemical parameters as well as the enzyme assays like SGPT, SGOT, ALP showed no significant change with those of the positive control group (PC) containing normal animals. Findings on the body weight, organ histology, serum biochemical parameters like serum, like triglycerides, cholesterols, ALP, creatinine, HDL-D, LDL-D, SGPT, and SGOT have shown significant similarities with the previous studies done with other established herbal medicines and it can be postulated that the *Aloe vera* gel at a dose up to 4 g/kg b.w. do not elicit any toxic effect in the body of the model animal when orally consumed for an extended period of time,

4.1.3. MTT assay

In the MTT assay, both peritoneal macrophage and spleenocyte cultures of M2 groups showed higher cell cytotoxicity than that in the M1 groups. Spleenocyte culture

showed an increase in cytotoxicity in a dose-dependent manner compared to PC (**Fig. 4.4, A** and **B** respectively).

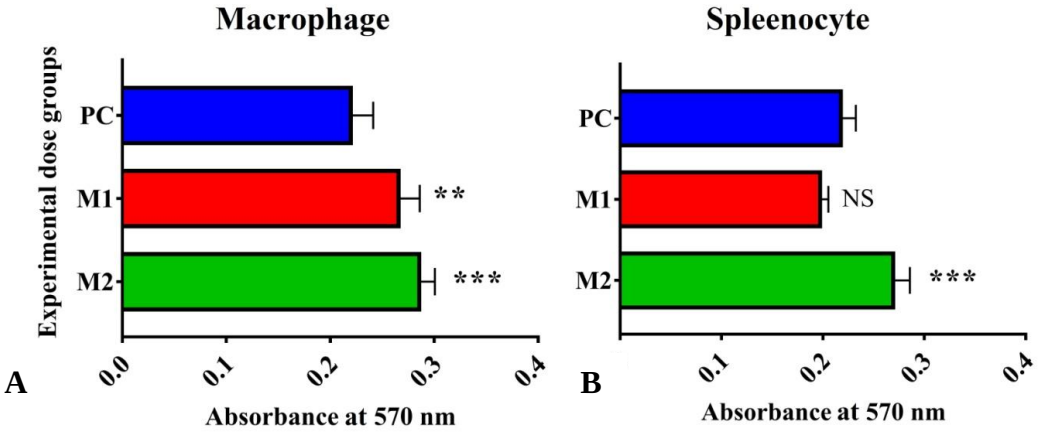


Figure 4.4. MTT assay of rat peritoneal macrophage (A) and spleenocyte (B) when treated with *Aloe vera* gel homogenate. PC refers to positive control; M1 refers to cultured suspension containing 50 µl plant homogenate, containing 60 mg Aloe gel; M2 refers to cultured suspension 50 µl plant homogenate, containing 120 mg *Aloe* gel. NS = non-significant; * = P< 0.05; ** = P < 0.01; *** = P< 0.001.

The MTT cell-viability tests (**Fig. 4.4, A, B**) were done to examine the cytotoxic effects of *Aloe vera* crude gel on peritoneal macrophages and spleenocytes of experimental rats. In the MTT assay, it is evident that higher dose of plant extract added to the cell cultures increased the death rate of the cells in the culture At a lower dose (dose group containing 30 mg of *Aloe* gel), the experimental group did not show any change in the cell viability (Data not shown). However, the experimental process and the dose groups used for MTT assay (M1 and M2) were much different from the *in vivo* experiments. The amount of *Aloe* gel added to the culture was strikingly higher from that of the *in vivo* studies. In the *in vivo* studies, following the oral administration of the plant gel and its absorption in the body system, the bioavailability of the bioactive compounds of the gel would be significantly low. Along with that, considering the number of body cells (in the *in vivo* studies) and the number of cultured macrophages or spleenocytes (in the *in vitro* MTT assay), the bioavailability of the plant bio-molecules will differ up to a large extent. That has probably been an important factor resulting in the absence of toxic effects of the *Aloe vera* gel in *in vivo* studies when administrated orally at orally consumable doses.

The MTT assay is a sensitive and reliable colorimetric approach to measure cell viability against a drug like compound. Plants like *Acanthospermum hispidum*, *Boerhavia diffusa*, *Croton gratissimus*, *Ficus sur*, *Spondias mombin* (Ogbole *et al.*, 2017), *Aspilia Africana*, *Andrographis paniculata* (Ala *et al.*, 2018) etc and many more plants with medicinal properties have been assessed for their role against cell viability through MTT assay. Plants recognized with anti-cancer potential generally show a high cell death in MTT assay at low extract dose. However, the present work nullifies the cytotoxic effect of *Aloe vera* gel at orally consumable doses up to 50 gm/ kg b.w.

4.1.4. Molecular docking studies for toxicity determination

In silico approach for docking of Aloe-derived phytocompounds with the major toxicity marker enzymes was considered to complement the idea of enzyme-inhibition by the Aloe-compounds which would lead to a change in the physiology of the model animal. Aloe-derived phyto-compounds were selected through literature study (section 3.2.2.2.4) and were simulated for molecular docking with the marker enzymes of the model animal body with the help of Autodock vina.

In the molecular docking analysis, the combination of phyto-components of *Aloe* with the selected proteins, showing the best binding-affinity with the least root mean square deviation (RMSD) from zero was considered. For the docking studies, the proteins were made ready by adding polar water and by removing water molecules wherever required. Non-polar hydrogens were also merged. All the bonds and torsions were left free to rotate. The computer simulated process found all the probable binding sites of the individual proteins with each of the selected phyto-compounds of Aloe gel and the binding energy was measured by the software. Among the selected proteins, glutamate dehydrogenase showed the substantial binding affinity with all the phyto-chemicals on average. This was followed by hepatitis Bx, which was considered as a model protein for the experiment. When the interactions with respect to ligands (phyto-compounds) were considered, Aloe-emodin proved to be the best ligand, followed by cholestanol. Aloe-emodin also had a strong binding affinity of -8.6 kcal/mol with the standard control hepatitis Bx (**Fig. 4.5, A** and **B**) during simulated interaction. The second-best interaction of Aloe-emodin was observed with

glutamate dehydrogenase, with a binding affinity of -8.5 kcal/mol (**Fig. 4.5, C** and **D**). When binding with Cox-2 was examined, Aloe-emodin displayed a binding affinity of -8.0 kcal/mol; second best binding was shown by cholestanol, which had a binding affinity of -6.5 kcal/mol (**Fig.4.6**). The rest of the target proteins and aloe-derived phyto-compounds showed different degrees of binding affinities which has been represented in a heat-map image (**Fig. 4.7**).

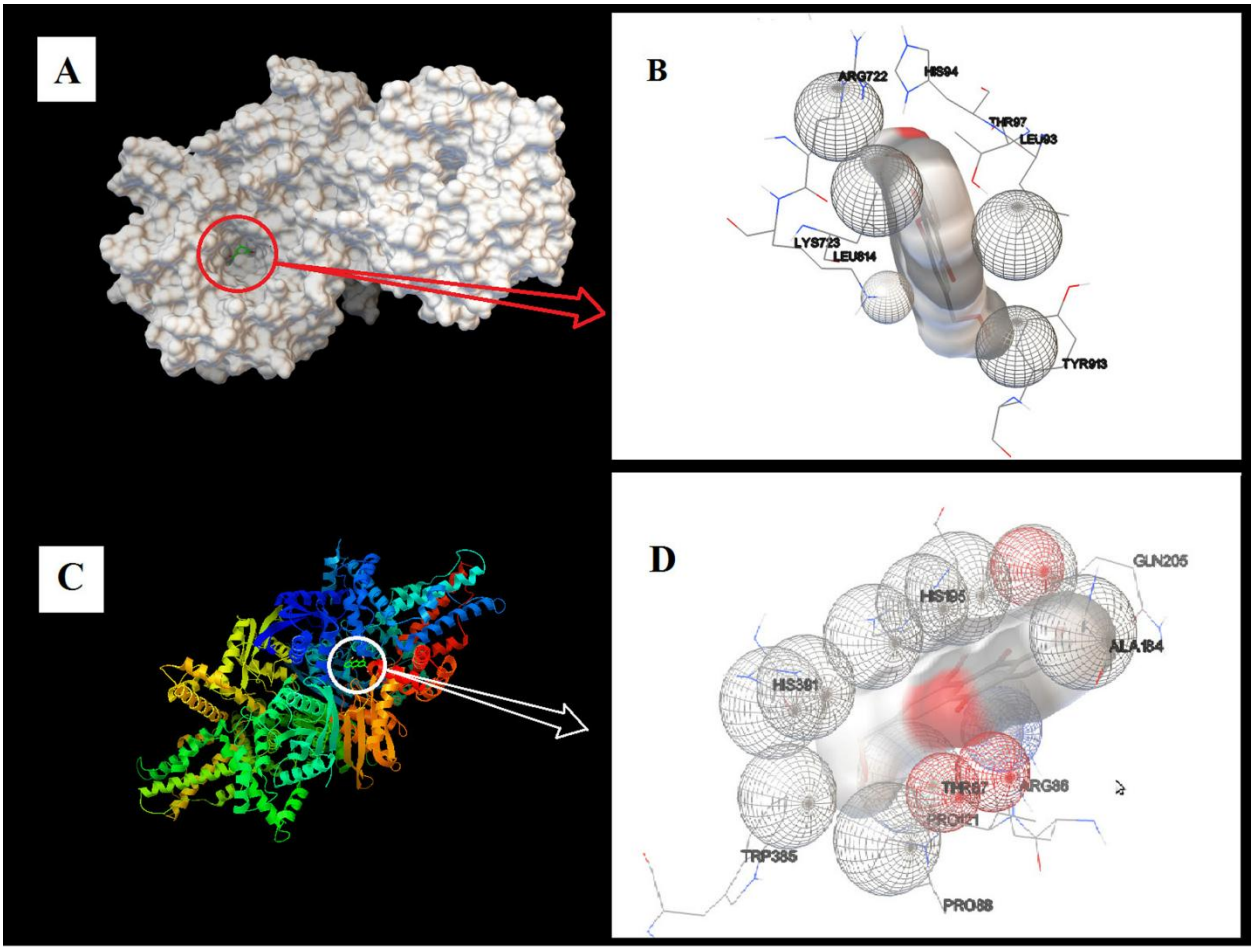


Figure 4.5. [A] Molecular docking of Aloe-emodin with hepatitis BX protein, the encircled area showing the active binding site of the protein with which the phytocompound interacts. [B] Details of the amino acids of hepatitis BX protein interacting with aloe-emodin (LEU93, HIS94, THR97, LEU614, ARG722, LYS723, TYR913). [C] Docking of Aloe-emodin with glutamate dehydrogenase protein, encircled region showing the position of aloe-emodin. [D] Positioning of amino acids of glutamate dehydrogenase protein interacting with aloe-emodin (ARG86, THR87, PRO88, PRO121, ALA184, HIS195, GLN205, TRP385, HIS391).

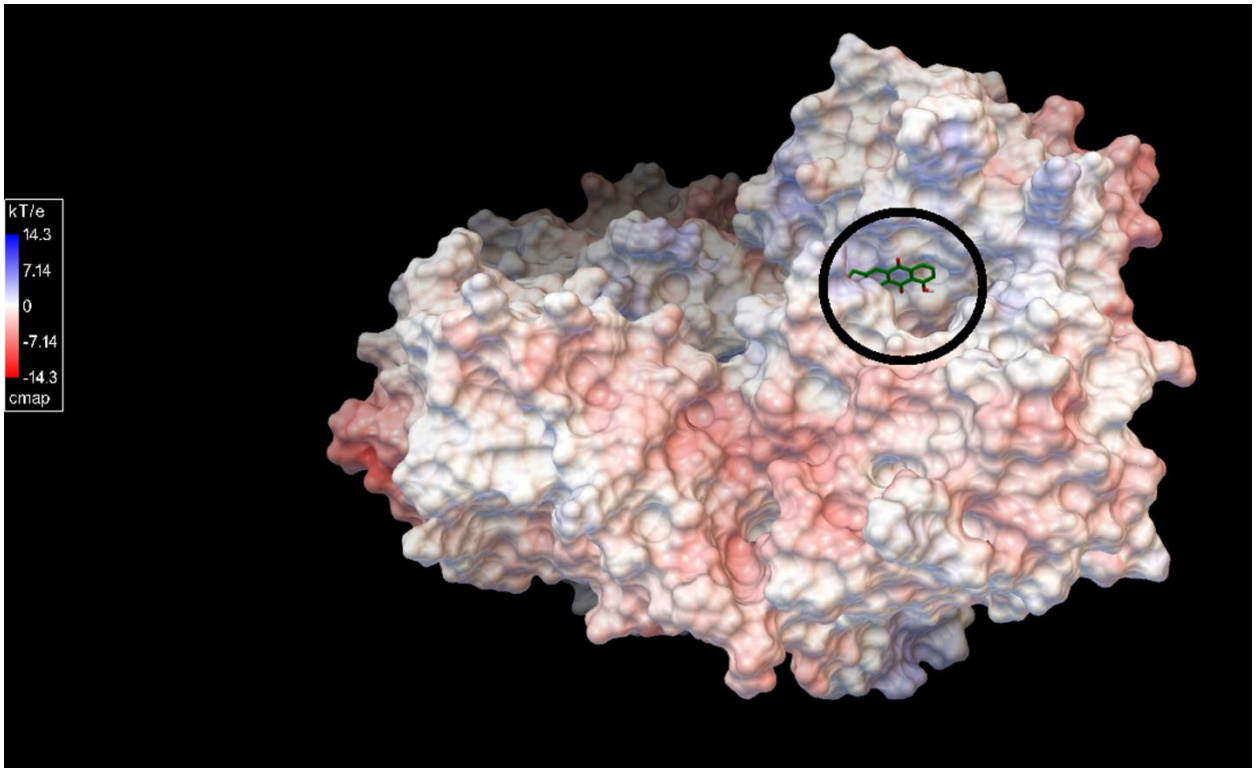


Figure 4.6. Simulated docking image of Aloe-emodin interacting with Cox-2. Encircled region indicates the best binding site.

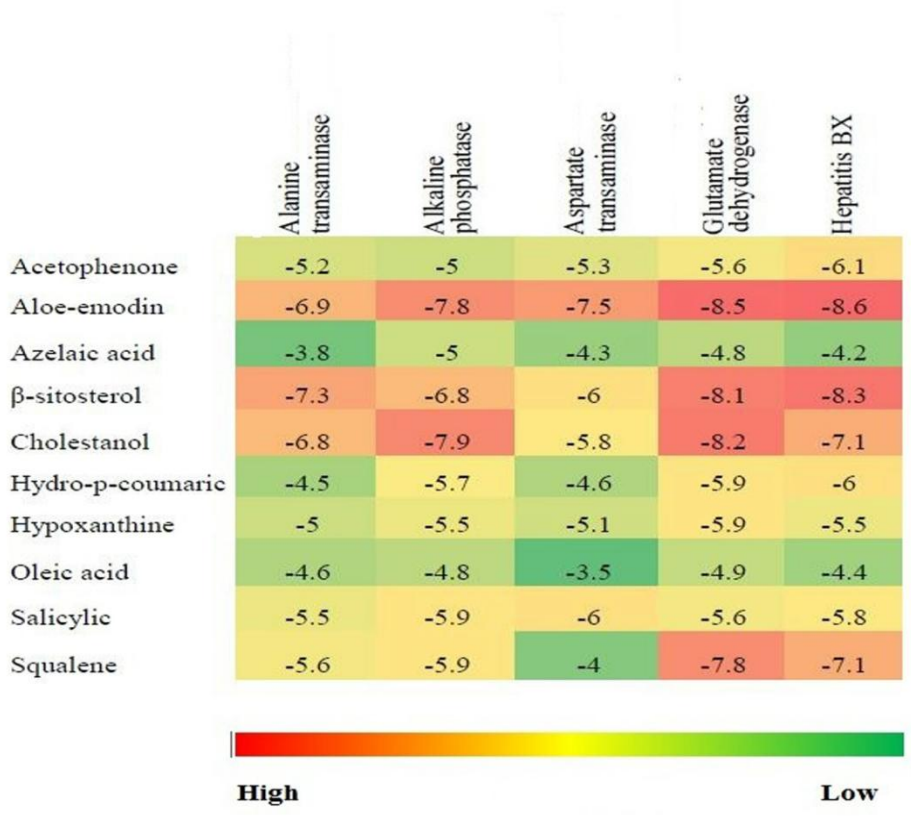


Figure 4.7. Heat-map showing the binding strengths (Kcal/mol) of different *Aloe vera* gel-derived pure phyto-compounds against the different toxicity-marker enzymes. Aloe-emodin showed the best binding strength with hepatitis BX followed by glutamate dehydrogenase enzyme.

The *in vivo* experimental parameters were further supported by the *in silico* binding or docking studies of the active compounds of *Aloe vera* gel with the enzymes of interest which were pre-selected from the target enzymes of the serum biochemical tests. The identified *Aloe vera* gel bio-active compounds were chosen from available scientific documentations and the promising compounds were selected for this study. Glutamate dehydrogenase, an important regulator of the urea cycle and a liver function indicator showed the best interactions with the bio-constituents of *Aloe vera* gel, particularly with Aloe-emodin (**Fig. 4.5, C** and **D**), cholestanol and β-sitosterol. The simulated interaction of standard protein hepatitis Bx also indicated an overall good interaction with all examined ligands including aloe-emodin. The best interaction was observed with Aloe-emodin (binding affinity of -8.6 kcal/mol) (**Fig. 4.5, A** and **B**). Hepatitis Bx is an indicator of liver dysfunction and during hepatitis, the up-regulation of this protein increases the chances of hepatic

carcinoma (Kew, 2011). As a part of the study, the strong interactions between *Aloe vera* gel constituents and the hepatitis Bx protein also indicates an inhibition of this protein during hepatitis. The strong binding between Aloe-emodin and Cox-2 raises a chance of Cox-2 down-regulation in the associated inflammatory pathway (Abdulla *et al.*, 2005) (**Fig. 4.6**). Our study also revealed numerous interaction sites for Aloe-emodin and other phyto-constituents with Cox-2 protein.

The other toxicological marker enzymes used for the *in silico* docking study (SGPT, SGOT and ALP) is the indicators of liver functioning. All the phytochemicals of the Aloe gel displayed average to moderate binding affinity with the proteins (**Fig. 4.7**). Phyto-compounds with a relatively high binding affinity are expected to hamper the normal functioning of the protein(s).

There are very scanty evidences about the *in silico* docking of plant extract derived phyto-compounds with the toxicity biomarker host body proteins. The present study represents a new perspective of research regarding the toxicity tests. However, plant derived purified compound like agathisflavone has been subjected to assess the toxicity and inflammatory effects *in silico* in a similar way. The compound showed strong binding affinity with both SGPT and SGOT proteins and it has been postulated that the binding of the compounds in the specific binding pocket of the enzymes with a high affinity would down-regulate the function of the enzyme (Andrade *et al.*, 2019). In the present study, the *in silico* molecular docking assay performed between *Aloe vera* gel phyto-compounds and toxic marker proteins suggests that the gel compounds can inhibit the expression of toxicity marker enzymes and thus, inhibit the toxicological outcomes.

4.1.5. Solubility analysis for toxicity and bio-availability

The *in silico* analysis of solubility provided an excellent information regarding the bio-availability of the phyto-compounds in the body system. The major phyto-components of the Aloe vera gel used in the molecular docking study were investigated for their solubility property which ensures the chances of their bio-availability. The process was done in computer simulated programme using ALOGPS and chemcalize online assistance. The structures of the phyto-components are assessed by the programmes to evaluate its solubility and absorption pattern in the blood plasma and other solvents. According to the theories of

octanol-water partition coefficient, log P should be between -0.4 and +5.6. Among the selected bio-active compounds of *Aloe vera*, β-sitosterol, cholestanol, hypoxanthine, oleic acid, and squalene remained outside the ideal range. The log S value is the parameter for solubility of the phytochemicals. The optimum range of log S value lies around -5 mol/L. *Aloe vera*-derived compounds, such as acetophenone, azelaic acid, hydro-p-coumaric, hypoxanthine and salicylic remained outside the range of good solubility. Another important solubility parameter is log D value, which has an ideal range of -3 to +3. Molecules with low log D values readily pass through the cell membrane and have better solubility. β-sitosterol, cholestanol, oleic acid and squalene were the compounds which were out of the preferred range (**Table 4.2**).

Table 4.2: Solubility analysis of the *Aloe* gel-derived pure compounds.

Chemicals	logP#	logS#	LogD#
Acetophenone	1.65	-1.95	1.53
Aloe-emodin	1.27	-2.96	1.88
Azelaic acid	1.37	-1.92	-3.65
β-sitosterol	7.27	-7.35	7.84
Cholestenol	7.02	-7.41	7.52
hydro-p-coumaric	1.15	-1.79	-1.28
Hypoxanthine	-0.74	-1.79	-0.42
oleic acid	7.68	-6.37	4.40
Salicylic	1.96	-1.09	-1.52
Squalene	8.64	-5.91	10.42

Log P is defined as the lipophilicity of the component resulting in the solubility of the compound in lipid medium.. Log S value is a unit stripped logarithm (base 10) of the solubility of a component in aqueous medium measured in mol/liter. log D, is the solubility of the phytocomponent at physiological pH

Bioavailability of the compounds are the important factors for effectiveness of the drug. Compounds with high binding affinity should also possess a proper solubility index to be available in the body to reach the cellular proteins (**Table 4.2**). Aloe-emodin qualified all the logP, logS, LogD thresholds and qualified as a potential ligand. The logD value considered at physiological pH of 7.4 ensured that Aloe-emodin could circulate through the plasma following absorption and could bind and interact with the target proteins. Aloe-emodin, a potent *anthraquinone* of *Aloe vera* origin, seemed to be the most promising compound for the bioactivity during toxicological studies.

4.2. RESULTS OF ANTI-INFLAMMATORY POTENTIAL

4.2.1. In vitro anti-inflammatory tests

4.2.1.1. Effect of *Aloe vera* gel homogenate on hypotonicity-induced hemolysis of hRBCs

The standard NSAID drug Indomethacin showed the highest protection (80.52±0.655%) against hypotonicity-induced RBC membrane lysis at the concentration of 200 µg/ml. *Aloe vera* gel homogenate protected the RBC membrane lysis in a concentration dependent manner **(Table 4.3)**. Maximum protective effect was seen in 1000 µg/ml dose of *Aloe vera* gel homogenate (74.89±1.26%). Results has shown that the *Aloe* gel-treated dose groups (600, 800, 1000 µg/ml) inhibited the RBC membrane hemolysis significantly in hypotonicity-induced stress **(Fig. 4.8, A)**.

4.2.1.2. Effect of *Aloe vera* gel homogenate on heat-induced hemolysis of hRBCs

All the experimental doses of *Aloe vera* crude gel homogenate (600, 800, 1000 µg/ml) elicited significant inhibition activities against heat-induced hemolysis of RBCs **(Fig. 4.8, B)**. The PI of hemolysis was dose-dependent in the experimental groups and maximum inhibition of hemolysis was observed in 1000 µg/ml *Aloe* gel dose group (20.86±0.770%). Although, standard NSAID drug Indomethacin showed the maximum inhibition of 43.98±1.52% at 200 µg/ml concentration **(Table 4.3).**

4.2.1.3. Effect of *Aloe vera* gel homogenate on protein-denaturation inhibition test

Aloe vera gel homogenate of different doses showed significant inhibition of protein denaturation in the dose dependent manner **(Fig. 4.8, C).** In the experimental groups, *Aloe* dose groups of 600 µg/ml, 800 µg/ml, 1000 µg/ml showed inhibition of 34.27±3.86%, 37.82±5.30%, 39.35±4.25% respectively. Diclofenac sodium, used as the standard NSAID drug, showed a PI of 46.74±1.84% at a concentration of 100 µg/ml **(Table 4.3).**

Table 4.3: Inhibitory properties of *Aloe vera* gel homogenate against hRBC membrane lysis and protein denaturation compared to their standard drug groups (Indomethacin, in membrane stabilization; and diclofenac sodium, in protein denaturation experiment).

Test samples	Concentration (µg/ml)	Mean % of inhibition ± S.E.M.		
		Hypotonicity induced hemolysis	Heat induced hemolysis	Protein denaturation
Aloe vera crude gel homogenate	600	39.87±.685	17.12±0.998	34.27±3.86
	800	52.04±2.18	18.36±1.79	37.82±5.30
	1000	74.89±1.26	20.86±0.770	39.35±4.25
Indomethacin	200	80.52±0.655	43.98±1.52	-
Diclofenac	100	-	-	46.74±1.84

Erythrocyte membrane stability test is a widely accepted study to assess the possible anti-inflammatory effect of synthetic drugs and traditional herbal remedies (Shinde *et al.*, 1999)**.** During inflammation, lysis of the lysosomal vesicle membranes releases their component enzymes to initiate the inflammatory response**.** A stabilized membrane prevents the release of its contents and inhibits inflammation. NSAIDs exert their effects by stabilizing the lysosomal membranes or by inhibiting the release of lysosomal enzymes (Anisoke *et al.*, 2012).

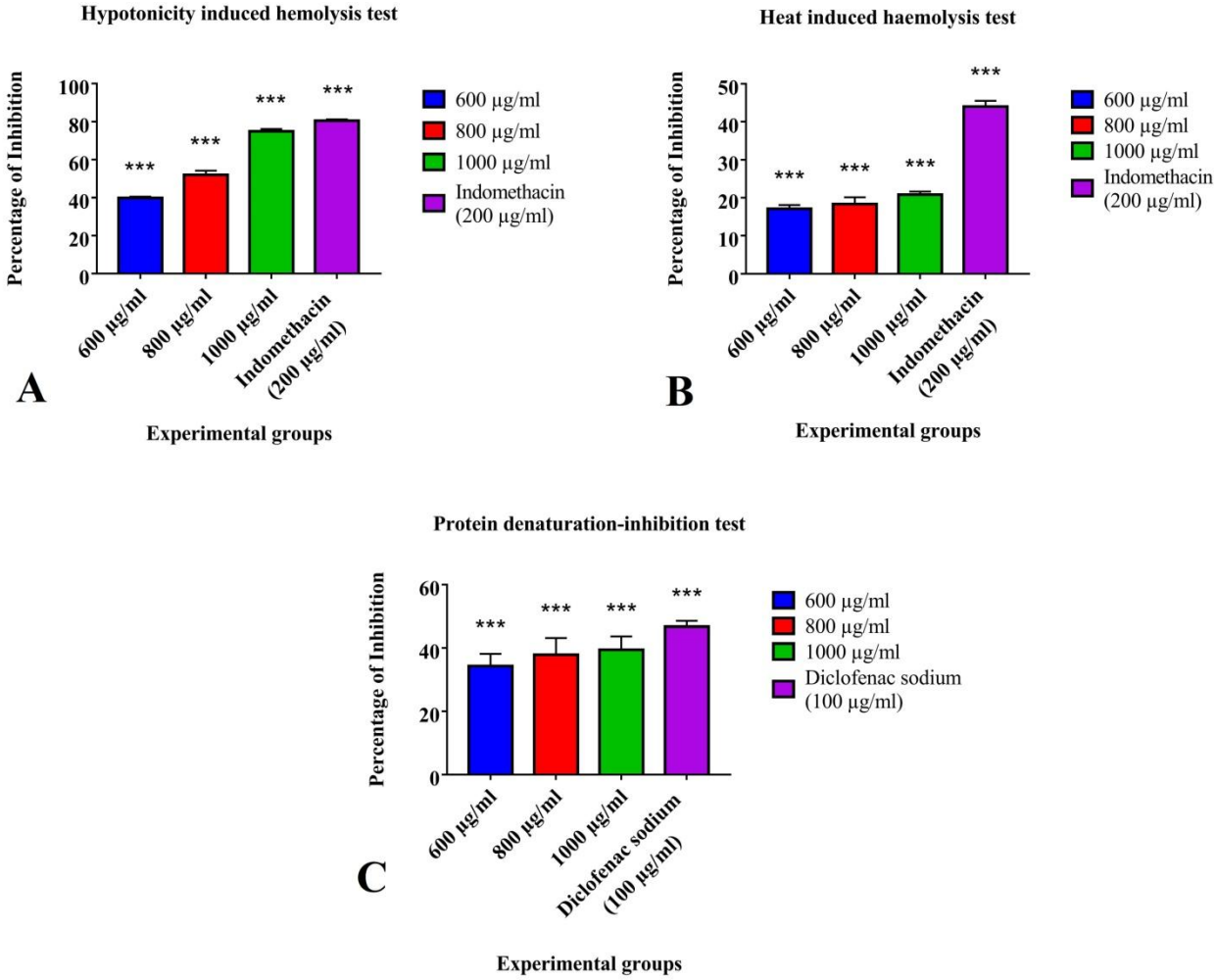


Figure 4.8. Figure representing the inhibitory role of *Aloe vera* gel against the hypotonicity-induced (A), heat-induced (B) haemolyses and against protein denaturation (C). The bar labels represent the different experimental dose groups and standard drug group. Significance value at P≤0.001 is indicated as ***.

The RBC is considered to be a model system for the study of lysosomes. Lysosomes have a great role in the elevation of inflammation in the inflamed site by releasing its lysosomal components and enzymes. If the lysosomal membrane is stabilized, the release of such inflammatory mediators is inhibited resulting in the down-regulation of inflammation. An exposure of RBCs to hypotonic medium or to elevated temperatures results in the lysis of the RBC membranes proceeded with hemolysis and hemoglobin oxidation. In the hypotonic

solution, excessive accumulation of fluid within the cell results in the rupture of its membrane**.** Increased body temperature also results in the rupture of the RBC membrane and hemolysis (Aloni *et al.*, 1977). If RBC membrane lysis (induced by heat or hypotonicity) is lowered by the subject drug-like compound, it is expected that the lysosomal membrane protection will be provided by the same subject *in vivo*. In the present study, a dose-dependent relationship of *Aloe vera* gel homogenate against both hypotonicity and heat-induced lysis of RBC membrane (**Fig. 4.8, A** and **B; Table 4.3**) has been found. Therefore, it can be postulated that all the experimental doses of plant extract can inhibit the release of lysosomal content during the inflammatory processes. A possible explanation for the membrane stabilizing activity of plant extracts could also depend on the increased surface area/volume ratio of the cell (Abe *et al.*, 1991) or the stabilization of the skeletal proteins such as tropomyosin by the phyto-compounds (Chasis and Mohandas, 1986; An *et al.*, 2007).

Protein denaturation is a well-documented feature in the advanced stages of chronic inflammatory diseases like RA (Rowley *et al.*, 1986). In the protein denaturation-inhibition test, treatment of egg albumin with increased heat resulted in the denaturation of egg albumin. Heat exposure causes the breakage of the functional three-dimensional (3-D) structure of protein. Standard NSAID drugs can inhibit heat-induced protein denaturation (Saso *et al.*, 2001)**.** In the present study, it has been found that *Aloe* gel homogenate inhibits the heat-induced protein denaturation at the experimental doses (**Fig. 4.8, C; Table 4.3**).

The screening of anti-inflammatory properties of the plant extracts and herbal remedies through the hemolytic assays and protein denaturation assay has been well practiced for over a decade now (Shinde *et al.*, 1999). The plants like *Cissus multistriata* (Omale and Okafor, 2008), *Albuca setosa* (Umapathy *et al.*, 2010), *Solanum aethiopicum* (Anisoke *et al.*, 2012), *Jatropha gossypifolia* (Nagaharika and Rasheed, 2013), *Piper chaba* (Yesmin *et al.*, 2020) has shown promising membrane stabilization activity and inhibition of protein denaturation and proved to be efficient anti-inflammatory CAM remedy in the traditional practice. From the results of *in vitro* anti-inflammatory experiments, it is found that *Aloe vera* gel homogenate shows no different anti-inflammatory potential when compared with such anti-inflammatory herbs.

4.2.2. In vivo anti-inflammatory tests

4.2.2.1. Cotton pellet induced granuloma formation test

The mean value of dried cotton pellet-induced granuloma was 0.051±0.006 gm in the standard group (STN). In the LD and HD groups, the mean dry weights of cotton pellet granulomas were 0.053±0.007 and 0.057±0.005 gm respectively. PC had the mean weight of 0.062±0.007 gm (**Fig. 4.9**). It has been estimated that, the LD group showed a 14.51% decrease in the dry-weight of cotton pellet, whereas HD showed 8.06% decrease against that of the PC. The STN group treated with indomethacin showed 17.74% decrease in the dry weight of cotton pellet compared to PC.

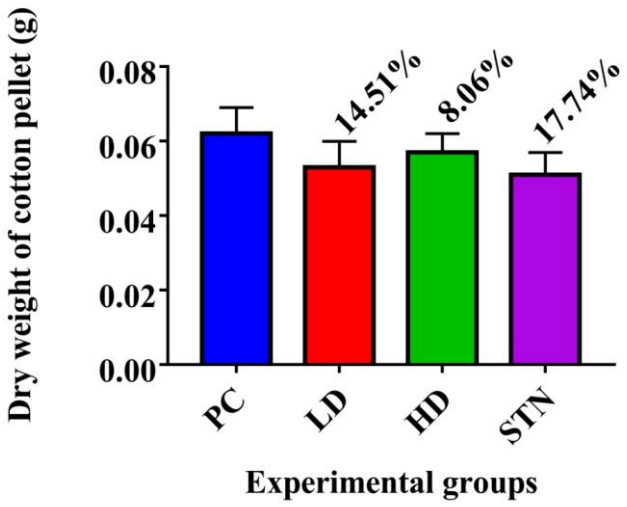


Figure 4.9. Diagram showing the dry-weight of cotton pellet-induced granuloma in different experimental groups. PC refers to positive control; LD and HD are experimental groups. LD corresponds to 0.40 g of *Aloe vera* gel homogenate / kg b.w. and HD correspond to 0.80 g of *Aloe vera* gel homogenate / kg b. w. The top of the bars express the PI in % compared with PC.

The cotton-pellet induced granuloma-formation model is an established model for the assessment of chronic inflammatory reaction (Sengar *et al.*, 2015). The granuloma formation against implantation of cotton pellet is characterized by three phases. Initial phase is the transudative phase which is early response generated within 3 h after cotton pellet implantation. It is characterized by increased vascular permeability which leads to leakage of fluids from blood vessels. The next phase is the exudative phase which continues from 3 h to about 72 h after implantation and it is characterized by protein leakage around the cotton

pellet where granuloma formation starts. It is a repairing mechanism against abnormally increased vascular permeability of the previous phase. The third phase is the proliferative phase which lasts from 3 days to 6 days and more, leading to the formation of granuloma tissue as a result of the release of pro-inflammatory mediators (Swingle *et al*., 1972; Pingsusaen *et al.*, 2015). In the transudative phase, increase in the wet weight of the cotton pellet occurs; whereas in the proliferative phase, increase in the dry weight of granuloma is observed (Damre *et al.*, 2003). Granuloma tissue formation is a result of increased in number of fibroblasts, synthesis of collagen and mucopolysaccharides, penetration of proliferating fibroblasts into the cotton pellet implanted region together, which ultimately leads to the formation of a vascularized mass (Hosseinzadeh *et al*., 2003). From the results, it is clearly seen that oral administration of *Aloe vera* crude leaf gel homogenate can reduce granuloma formation (**Fig. 4.9**). It was found that the extract elicited significant anti-inflammatory activities in both LD and HD experimental group rats by inhibiting the migration of immune cells and reducing the dry weight of granuloma.

The experiment has been implemented on model animals using different other plant extracts to validate their role against inflammation. Some recent works include the plants like *Agave Americana* (leaf extract at a dose of 400 mg/ kg b.w.) (Mishra *et al.*, 2018), *Stemonocoleus micranthus* (stem bark at a dose of 400mg/ kg b.w.) (Mbaoji *et al.*, 2020), *Bryophyllum pinnatum* (leaf part at a dose of 400 mg/kg b.w.) (Oladejo *et al.*, 2021) etc. A few studies have also quantified the major inflammatory marker proteins for this inflammatory model and the inflammatory markers were down-regulated when the inflamed model animal was treated with plant extract (Oladejo *et al.*, 2021). All the plants show the reduction in granuloma weight which is very similar to our recent finding on *Aloe vera* gel homogenate. So, it can be concluded that the unprocessed orally consumed *Aloe vera* gel has substantial potent anti-inflammatory activity which inhibits the formation of granuloma in experimental animals.

4.2.2.2. Carrageenan-induced paw swelling test

It is evident that in the standard (STN) group, indomethacin treatment showed the best PI of 92.7% after 3.5 hours (210 min) of carrageenan injection when compared against

NC. Experimental groups LD and HD also effectively decreased paw swellings by 58.69% and 74.09% respectively. (**Fig. 4.10**).

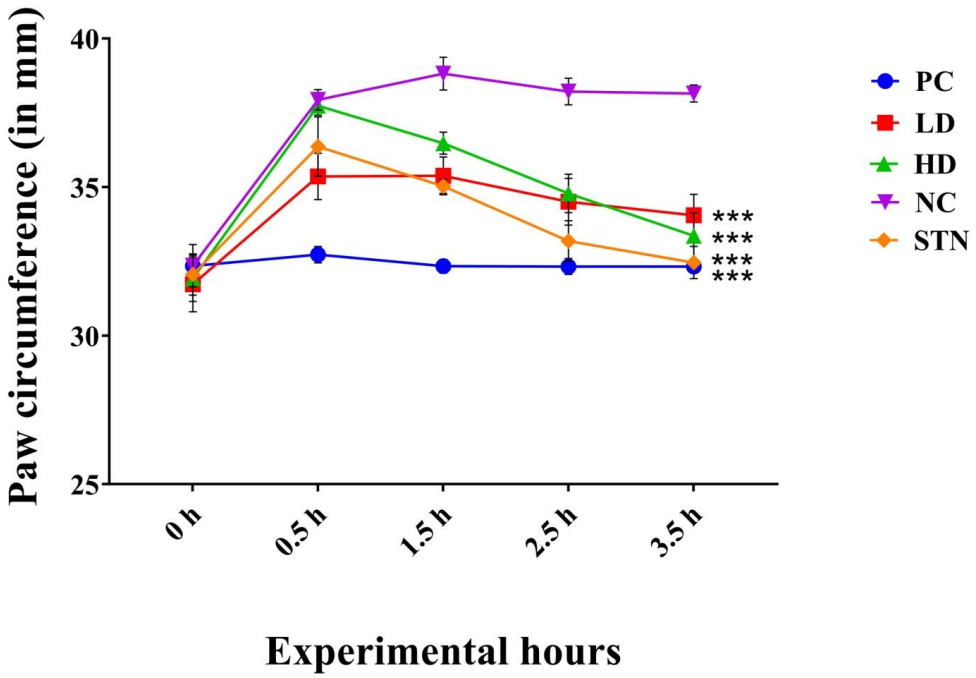


Figure 4.10. Carrageenan-induced paw swelling (measured as paw circumference in mm) in different animal groups expressed as mean values with respect to time (in hour). PC refers to positive control; LD and HD are experimental groups. LD corresponds to 0.40 g of *Aloe vera* gel homogenate / kg b.w. and HD correspond to 0.80 g of *Aloe vera* gel homogenate / kg b. w. NC refers to negative control group. STN refers to standard drug group in which indomethacin was used at a dose of 10 mg/kg b. w. NS = non-significant; * = P< 0.05; ** = P < 0.01; *** = P< 0.001.

Carrageenan-induced paw edema is another established model for *in vivo* study of anti-inflammatory activity of drug-like products. The early phase of inflammation following carrageenan induction is initiated by histamines and serotonins. The tissues of the region exposed to carrageenan also show increased prostaglandin synthesis when damaged (Gupta *et al.*, 2006). The late phase is initiated by bradykinins, polymorphonuclear immune cells and prostaglandins released from invading macrophages (Gupta *et al.*, 2006). Prostaglandins help in the elevation of the temperature of the inflamed region and cause inflammatory pain. NSAID drugs like indomethacin (standard drug used in the experiment) inhibits the enzyme prostaglandin H2 synthase (also known as cycloxygenase or Cox) which is a catalyst of prostaglandin synthesis in the early steps. Inhibition of inflammation is measured by the

inhibition of paw-swelling in the carrageenan-induced paw edema model. It is also postulated that the inhibition in inflammation occurs as the phyto-constituents of the *Aloe vera* gel suppresses the release of inflammatory mediators including histamine, serotonin, bradykinin and prostaglandins which are responsible for the acute inflammation.

This study has found that the *Aloe vera* gel homogenate down-regulate the inflammatory swelling caused by carrageenen in the model animal in a dose-dependent manner by interacting with one of the above mentioned pathways. In this respect, *in silico* docking of Cox-2 protein with *Aloe* gel phyto-compounds like Aloe-emodin during the toxicological analysis also revealed the similar result indicating a strong possibility of Cox-2 inhibition by *Aloe vera* gel (**Fig. 4.6**).

The well explored medicinal plants like *Terminalia bellerica* (Chauhan *et al.*, 2018), *Muntingia calabura* (Jisha *et al.*, 2019), *Anacardium occidentale* (Cordaro *et al.*, 2020), *Xanthium indicum* (Chaudhari *et al.*, 2020), *Stemonocoleus micranthus* (Mbaoji *et al.*, 2020) have also shown significant inhibition of carrageenan-induced paw edema in the model animals. The present study therefore confirms the anti-inflammatory role of the naturally harvested crude unprocessed *Aloe vera* gel in this regard.

4.3. ANTI-ARTHRITIC POTENTIAL ASSESSMENT

4.3.1. FCA-induced arthritic rat model

4.3.1.1. Biometric studies

- **Measurement of paw circumference**

The FCA induced inflammatory arthritic rat model was selected for the assessment of anti-arthritic property of the naturally harvested unprocessed *Aloe vera* gel. The animals were injected with FCA in the hind paw which elicited arthritis-like conditions in the animal hind paw joint. The paw-circumference, body weight were monitored regularly and joint histology and haematological parameters were monitored at the end of 28 day long *Aloe* gel feeding schedule. Paw circumference was measured using a vernier caliper as mentioned in the Material and Method section (section 3.2.4.1.1). After the induction of arthritis by injecting FCA, the paw swellings were achieved in all the groups within 3-4 days (**Fig. 4.11 A** and **B; Day 3**). In the following days, paw-swelling in *Aloe*-gel treated groups decreased and approached normalcy around the 10th day. The FCA group rats retained more paw swelling

compared to the *Aloe*-gel treated experimental groups (LD and HD). The same feature was also observed following the booster injection with FCA. There was significant increase in the mean value of paw circumference (in mm) in the entire FCA-induced arthritic groups (LD, HD and FCA) (**Fig. 4.11**). But the paw-circumference of the plant gel-treated experimental groups (LD and HD) showed significant reduction in the increment rate of paw-swelling during treatment with *Aloe vera* gel homogenate. Both the LD and HD groups showed 23.30% and 65.59% less swelling respectively, compared to the FCA group on the 28th day. Similar studies conducted on *Cinnamomum zeylanicum* (Vetal *et al.*, 2013), *Barleria prionitis* (Choudhary *et al.*, 2014), *Berberis calliobotrys* (Hasan *et al.*, 2015) and so other plants (Ekambaram *et al.*, 2010) have shown a similar pattern regarding the reduction of paw-swelling when treated with subject plant extract.

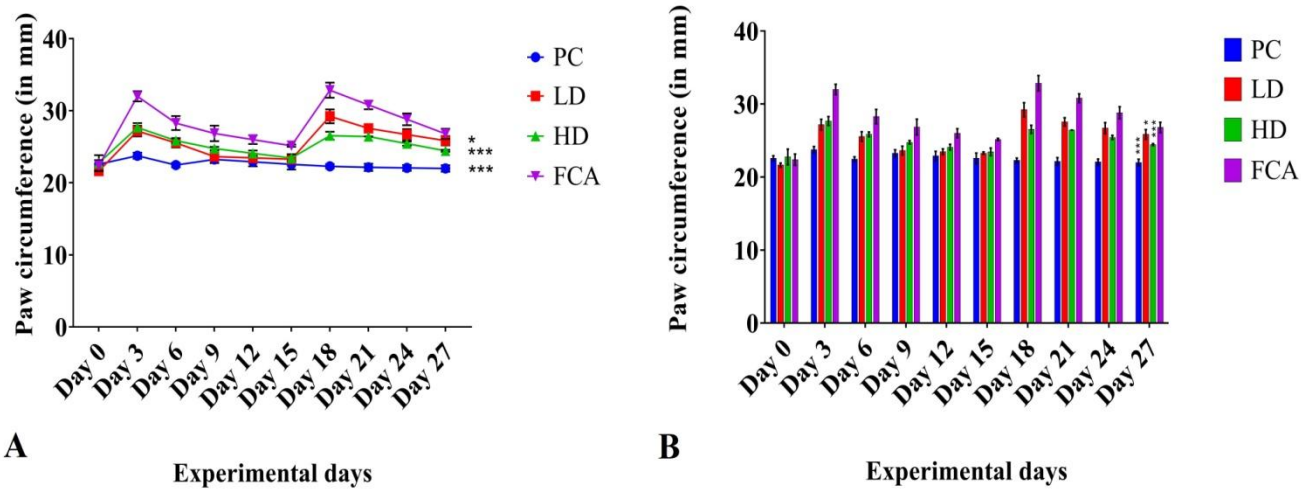


Figure 4.11. Comparison of rat paw circumference of different groups (in mm) following FCA injection (A) represents the line diagram showing the patterns of changes in the paw circumference through the experimental days up to 28th day. (B) represents day-wise measurement of the paw circumference in different groups in different experimental days expressed in bar diagram. PC refers to positive control; LD and HD are experimental groups. LD corresponds to 0.40 g of *Aloe vera* gel homogenate / kg b.w. and HD correspond to 0.80 g of *Aloe vera* gel homogenate / kg b. w. FCA refers to arthritic control group. NS = non-significant; * = P< 0.05; *** = P< 0.001.

- **Photograph and radiological imaging of paw joint:**

The photographs of the FCA-injected hind paws of the different rat groups were captured on the 21st and 28th day of the experiment. Following the anesthesia-based sacrifice of the animals, the separated hind paws were subjected to radiological analysis. Radiological imaging as well as the photographs confirmed dilation of the paw joints in all the induced-arthritis groups at the 21st and 28th days of experiment commencement. However, when the images were compared, the FCA group clearly showed more dilation in the joint structure compared to the LD and HD group animals (**Fig. 4.12**). Clearly, the Aloe gel treated group showed decrease in the paw-swelling and joint deformities were less. The degree of swelling of the inflamed paw was confirmed by the estimation of paw circumferences in different animal groups (**Fig. 4.11**); radiological images were compared and lack of integrity of the paw joint was well evident. Conclusions from both the photographic and radiological data were further confirmed through a blind-folded checking by a subject expert, where the animal groups were hidden at the time of checking by the expert.

The occurrence of joint space-reduction or narrowing of joint spaces between bones in arthritic models have been shown by other authors where herbal remedies like *Strychnos potatorum* (Ekambaram *et al.*, 2010), *Tridax procumbens* (Petchi *et al.*, 2013), *Sesamum indicum* (Ruckmani *et al.*, 2018), *Berberis calliobotrys* (Hasan *et al.*, 2021) have significantly reduced the joint damage when these complementary medicines are orally supplemented in the model animals. The present study on *Aloe vera* gel has shown its potential bone-joint protection and healing properties when orally fed in the inflamed arthritic model animals.

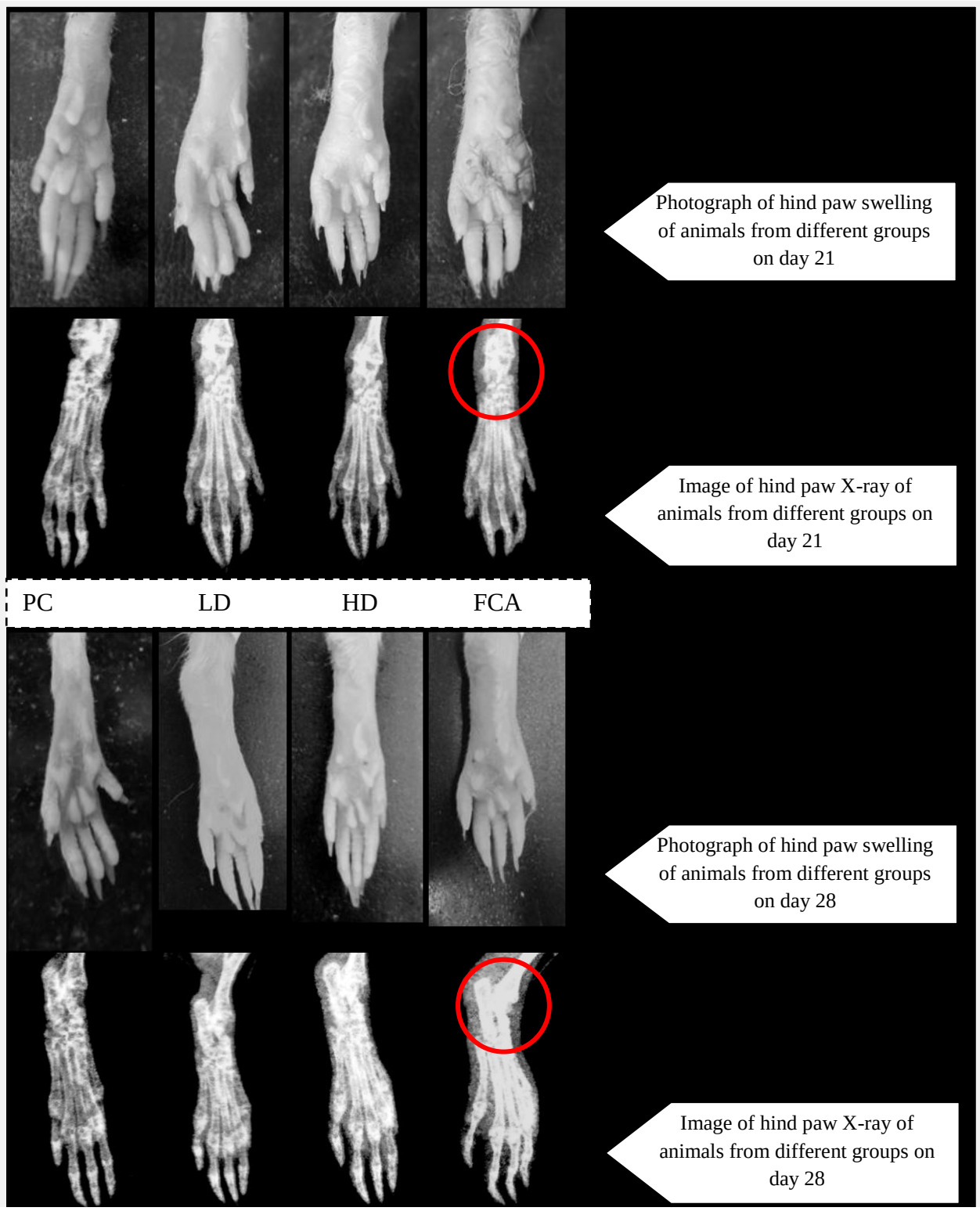
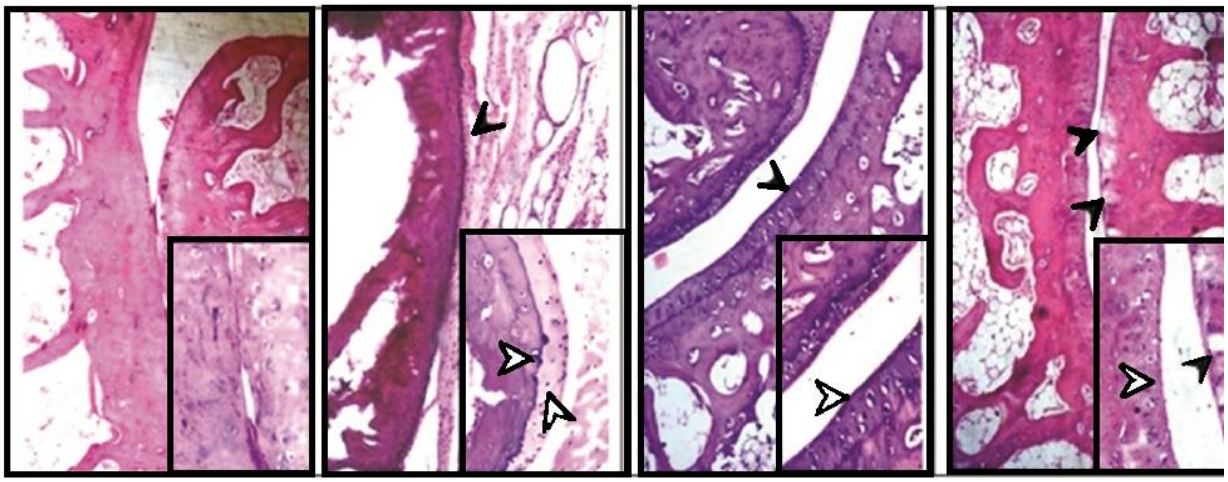


Figure 4.12. Photographs and X-rayed images of FCA-injected hind paws from different animal groups on both 21st and 28th day of the experiment. In the photographs, the degree of paw swelling can be visualized by the lack of prominence of the foot pads. In the X-ray images, the degree of integrity of the joints is visible. The encircled regions of FCA group shows lack of structural integrity leading to a less clear image formation. PC refers to positive control; LD and HD are experimental groups. LD corresponds to 0.40 g of *Aloe vera* gel homogenate / kg b.w. and HD correspond to 0.80 g of *Aloe vera* gel homogenate / kg b. w. FCA refers to arthritic control group.

- **Histological studies:**

Histological observations showed that the cartilage layer of LD and HD group animals were less affected compared to the cartilage layer of FCA group animals. The surface layers of the cartilage were less irregular in the experimental groups (LD and HD) where Aloe gel was used as the remedy. The immune cell infiltration was more in the joints of FCA group animals compared to LD and HD (**Fig. 4.13**; cellular infiltration indicated by white arrows). Histological observations also clearly showed that the gel-treated (LD and HD) groups had less cellular infiltration in the cartilage compared to the negative control (FCA) (**Fig. 4.13**). The structure of the cartilage layer showed more tears and uneven lining in the FCA group rat joints (**Fig. 4.13**; irregular cartilage layer and decreased joint space in indicated by black arrows). These observations confirmed that the cartilage layer is protected when treated with *Aloe vera* gel homogenate. In the similar works done by Ekambaram *et al.* (2010), Choudhary *et al*. (2014); researchers have considered the histopathological assessment of the bone joint as a major parameter as this provides a strong visual interpretation of data for the determination of the efficacy of herbal remedies against inflammatory arthritis. These works have shown similar findings where the histopathology of the bone joint of the plant extract treated inflamed model animal showed restoration of the joint structure.



PC **LD** **HD** **FCA**

Figure 4.13. Longitudinal sections of the hind paw-joints of different rat groups on the 21st day of the experiment. The white arrows indicate the immune cell infiltration in the cartilage region; the black arrows indicate the damage and degradation of the cartilage leading to less stained regions. Insets indicate 40X magnification within a 10X magnified image of bone joints. PC refers to positive control; LD and HD are experimental groups. LD corresponds to 0.40 g of *Aloe vera* gel homogenate / kg b.w. and HD correspond to 0.80 g of *Aloe vera* gel homogenate / kg b. w. FCA refers to arthritic control group.

4.3.1.2. Hematological studies

In both 21st day and 28th day of experimental commencement, the experimental animals showed decrease in RBC counts in arthritic condition (FCA group) compared to PC (5.43 ± 0.485 and 6.24 ± 0.693 $\times 10^6$ RBC/mm^3 respectively in 21st and 28th day for FCA group; and 9.27± 0.490 and 9.27 ± 0.732 $\times 10^6$ RBC/mm^3 respectively for 21st and 28th day for PC) (**Table 4.4**); in LD and HD, the RBC count increased significantly compared to the FCA group. RBC count for LD on 21st and 28th day of experiment remained 8.44± 0.674 and 8.88± 0.737 $\times 10^6$ RBC/mm^3 respectively; whereas RBC count for HD on 21st and 28th day remained 7.92 ± 0.442 and 8.84± 0.763 $\times 10^6$ RBC/mm^3 respectively (**Table 4.4**). It clearly states that the RBC numbers are towards restoration in arthritic animals when those were treated with *Aloe vera* gel homogenate. Total WBC count increased significantly in the FCA group (7.20 ± 0.489 and 6.90 ± 0.600 $\times 10^3$ WBC/mm^3 on 21st and 28th day) when compared to PC (5.100 ± 0.503 and 5.50 ± 0.683 $\times 10^3$ WBC/mm^3 on 21st and 28th day respectively). The treatment with *Aloe vera* gel homogenate in experimental groups brought back the parameter toward their normal levels. LD showed 5.70± 0.382 and 5.90 ± 0.600 $\times 10^3$

WBC/mm^3 on 21st and 28th day respectively whereas HD showed 6.20 ± 0.516 and 6.60 ± 0.765 ×10^3 WBC/mm^{3}on 21st and 28th days of experiment respectively (**Table 4.4**). Similar scenario was observed in the haemoglobin count of different animal groups on 21st and 28th days of experiment (**Table 4.4**). Compared to the PC group (11.6±0.56 and 12.8±0.58 g/dl on 21st and 28th day), FCA group showed a decrease in haemoglobin count (7.20±0.52 and 7.80±0.46 g/dl on 21st and 28th day respectively); the haemoglobin count was restored towards normalcy in *Aloe* gel treated LD (10.5±0.72 and 11.0±1.06 on 21st and 28th day respectively) and HD (11.2 ±0.79 and 10.2±1.08 on 21st and 28th day respectively) group animals in both experimental days (**Table 4.4**).

Table 4.4: Table representing the differences in the hematologic parameters of different experimental animal groups. RBC, Total WBC and hemoglobin count has been done on 21st and 28th day. NS = non-significant; * = P< 0.05; ** = P < 0.01; *** = P< 0.001

	RBC (7-10*10^6/mm^3)		**WBC (6-17*10^4/mm^3)**		**Hemoglobin (11-18 g/dl)**	
	21st day	**28th day**	**21st day**	**28th day**	**21st day**	**28th day**
PC	9270000 ± 490408	9270000 ± 732802	5100 ± 503	5550 ± 683	11.6± 0.56	12.8 ± 0.58
FCA	5430000 ± 485283 **a*** **	6240000 ± 693181 **a*** **	7200±489 **a*** **	6900±600 **a***	7.2±0.52 **a*** **	7.8±0.46 **a*** **
LD	8440000 ± 674907 **b*** **	8880000 ± 737054 **b*** **	5700 ±382 **b****	5900 ± 600 **b**	10.5± 0.72 **b*** **	11.0 ± 1.06 **b*** **
HD	7920000 ± 442436 **b*** **	8840000 ± 763544 **b*** **	6200±516 **b***	6600 ±765 **b**	11.2±0.79 **b*** **	10.2±1.08 **b****

Normal ranges of haematological counts in rats, according to CPCSEA, MoEF, India are given within parentheses. PC refers to positive control; LD and HD are experimental groups. LD corresponds to 0.40 g of *Aloe vera* gel homogenate/kg b.w. and HD correspond to 0.80 g of *Aloe vera* gel homogenate/kg b. w. FCA refers to arthritic control group. a = comparison made between positive control (PC) and other groups; b = comparison made between negative control (FCA) and other groups.

RBC count usually decreases in arthritic conditions. This is because the bone marrow loses its normal functioning and fails to respond to anemic condition (Mowat, 1971). The RBC count increased significantly in experimental groups after 21st and 28th days and

approached normalcy (**Table 4.4**). In contrast, WBC count is increased in arthritic condition. Triggering of the immune system with the FCA-antigens lead to the increased WBC in the circulation (Ekambaram *et al.*, 2010). The levels of WBC in experimental groups treated with *Aloe vera* gel homogenate reached the normal values and showed no significant difference with that of the positive control rats (**Table 4.4**). The hemoglobin count also showed similarity with the value of RBC, as low RBC count results in low hemoglobin levels. The experimental groups showed significant increase in hemoglobin when compared with the negative control group.

4.3.1.3. Serum Biochemical studies

For the determination of serum biochemical parameters, the blood was collected from the animals after killing the animals under proper anesthesia following animal ethical committee promoted guidelines. The collected blood from each of the animals was kept separately for the isolation of serum. The serum protein, serum albumin, ceruloplasmin and creatinine of all the animals from each group was quantified from each of the animals separately using manual methods or biochemical kits in spectrophotometer (section 3.2.4.1.3). Arthritic rats (FCA group) showed a significant decrease in the serum level of total protein that was restored towards the normal ranges after *Aloe*-gel treatment. In the FCA group, serum total protein decreased 4.430 ± 0.48 and 4.89 ± 0.15 g/dl on 21^{st} and 28^{th} day compared to PC, which showed 9.12 ± 0.50 and 9.87 ± 0.52 g/dl amount of serum total protein on 21^{st} and 28^{th} day of experiment. The LD and HD groups both showed an increase in total protein level after *Aloe* gel treatment. In LD group, the total protein restored up to 4.70 ± 0.10 and 5.28 ± 0.23 on 21^{st} and 28^{th} day of experiment respectively; in HD group, it restored up to 5.82 ± 0.16 and 5.89 ± 0.12 g/dl amount on 21^{st} and 28^{th} day of experiment respectively. (**Fig. 4.14, A** and **B; Table 4.5**). The experimental groups also showed restoration of the elevated serum ceruloplasmin level after feeding with *Aloe vera* gel homogenate (**Fig. 4.14, C** and **D**). Along with that, the experimental groups treated with *Aloe*-gel showed increased levels of serum albumin on 21st day than that in the diseased animals of negative control group. (**Table 4.5**). Arthritic rats showed an increase in serum creatinine level on the 21st day. However, the value of creatinine was normal in the *Aloe*-gel treated experimental groups (**Table 4.5**).

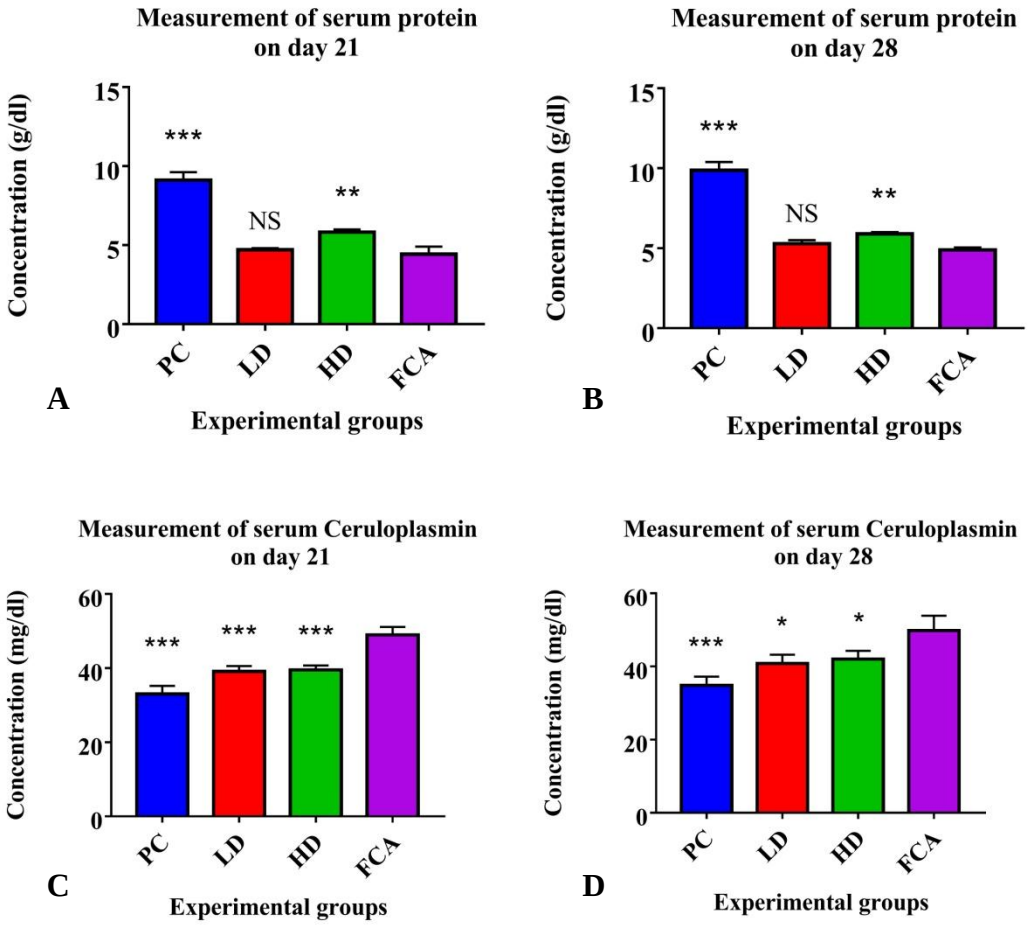


Figure 4.14. Estimation of serum protein and serum ceruloplasmin of different rat groups on 21st and 28th day after induction of arthritis through FCA injection. The FCA group data was compared with the rest of the groups. PC refers to positive control; LD and HD are experimental groups. LD corresponds to 0.40 g of *Aloe vera* gel homogenate / kg b.w. and HD correspond to 0.80 g of *Aloe vera* gel homogenate / kg b. w. FCA refers to arthritic control group. NS = non-significant; * = P< 0.05; ** = P < 0.01; *** = P< 0.001

Table 4.5: Measurement of serum protein, albumin and creatinine in different experimental groups.

	Total protein (g/dl)	Total protein (g/dl)	Albumin (g/dl)	Creatinine (mg/dl)
	21st day	**28th day**	**21st day**	**21th day**
PC	9.12±0.50	9.871±0.52	6.958±0.02	2.922±0.22
FCA	4.430±0. 48	4.89±0.15	3.312±0.06	4.856±0.07
LD	4.702±0.10	5.280±0.23	3.951±0.05	3.755±0.03
HD	5.821±0.16	5.891±0.12	3.730±0.02	2.970±0.06

PC refers to positive control; LD and HD are experimental groups. LD corresponds to 0.40 g of *Aloe vera* gel homogenate / kg b.w. and HD correspond to 0.80 g of *Aloe vera* gel homogenate / kg b. w. FCA refers to arthritic control group.

The level of total protein usually decreases during arthritis (Ekambaram *et al.*, 2010). Rats of the negative control or FCA group showed a significant decrease in the total protein level in comparison to the positive control rats in both the experimental time points (21st and 28th days). All the experimental groups showed the tendency to increase the protein level with statistical significance (**Fig. 4.14, A** and **B; Table 4.5**). It reflects the role of *Aloe vera* gel in restoring total protein level of serum during RA. When compared to the increment of the body weight in these treatment groups, the role of protein synthesis become an important factor in maintaining the body weight. Similar to the serum protein, the total serum albumin level is reported to decrease in arthritic condition. It can be postulated that the increased permeability of vascular cells during the arthritic inflammation may increase the diffusion of albumin into vascular tissue and thereby may decrease the level of albumin in the blood. The experimental group rats showed an increased albumin concentration when compared to the FCA group rats (**Table 4.5**). Ceruloplasmin is a major superoxide scavenger and increases during the rheumatoid arthritis condition (Ekambaram *et al.*, 2010). The experimental rat groups showed a restoration of elevated serum ceruloplasmin after the treatment with *Aloe vera* gel homogenate in both the 21st and 28th days (**Fig. 4.14, C and D**), confirming the

efficacious role of *Aloe vera* gel in the decrement of ceruloplasmin level. Arthritic rats also showed an increase in the serum creatinine level on 21st day. Serum creatinine concentration increased in the FCA group rats when compared to the positive control rats but all the other experimental group animals (LD and HD) showed no significant change when compared to the PC which indicated that the level of creatinine is normal (**Table 4.5**).

Other herbal remedies have shown similar results when the changes in the haematological and serum biochemical parameters were observed following the oral consumption of the subject plant in arthritic animals. Plants like *Strychnos potatorum* (Ekambaram *et al.*, 2010), *Tridax procumbens* (Petchi *et al.*, 2013), *Sesamum indicum* (Ruckmani *et al.*, 2018) showed promising activities where haematological and serum biochemical parameters were brought back to normalcy in model animals following oral supplementation of herbal extracts. Total protein, albumin and ceruloplasmin showed a tendency to increase and creatinine showed a tendency to decrease in all these previous studies.

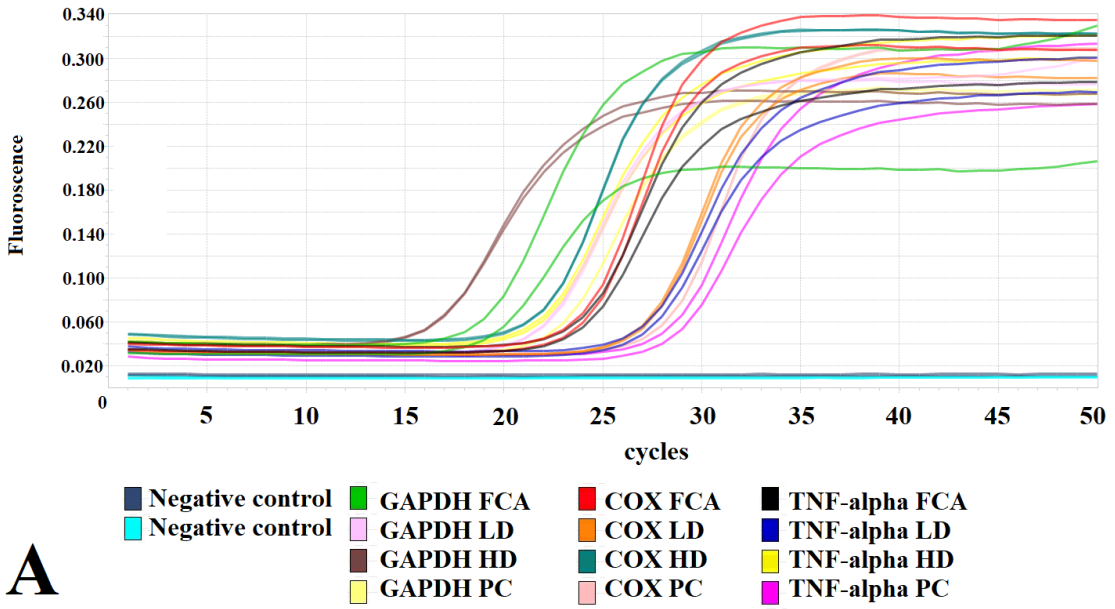
4.4. REAL TIME-QUANTITATIVE PCR METHOD FOR THE RELATIVE EXPRESSION STUDY OF SOME SELECTED CYTOKINES AND BIOMARKERS

For the real time quantification of TNF-α and Cox-2, the blood was collected from the animals separately from all the groups. As discussed in the section 3.2.5.1, animals from PC, FCA, LD and HD group were sacrificed on 28^{th} day of experiment and the blood collection was done. RNA was isolated from the bloods of each of the animals separately and were pooled together for each of the groups. The pooled RNAs were subjected to cDNA preparation and RTqPCR. The quantification of the genes was done by $2^{-\Delta\Delta Ct}$ method where the housekeeping gene or internal control gene (GAPDH) served as a calibrator. The degree of expression change of target genes compared to calibrator gene was estimated in each group and the result was obtained in the form of changes in fold of expression. The transcription level expression of the target and the internal control genes were quantified in the negative control (FCA) and experimental animals (LD and HD) compared to the positive control animals (PC). All the target and control genes showed Ct values at less than 30 (**Fig.**

4.15 A). The expression bar for the group PC is not shown in the bar diagram as in the relative expression study as the expression of a gene in positive control (PC) animal is considered to be 1 and the fold change in the gene expression in the experimental group animals are measured relative to that normal expression (**Fig. 4.16**).

The dissociation curve analysis showed three distinct dissociation temperatures for three distinct genes (**Fig. 4.15 B**; circle A represents dissociation temperature zone for TNF-α. Circle B represents dissociation temperature zone for GAPDH and circle C represents dissociation temperature zone for Cox-2 respectively).

Following the $2^{-\Delta\Delta Ct}$ method, TNF-α gene showed an elevated expression (expressed as fold change) in the FCA group (2.271±0.85) compared to PC (1-fold). TNF-α expression was reduced in both the *Aloe* gel treated groups (1.456±0.11 and 1.396±0.10 folds in LD and HD groups respectively) compared to FCA group. Cox-2 increased in FCA treated animals compared to the PC (1.842±0.68) but decreased in the treated groups (1.259±0.11 and 1.198±0.083 folds for LD and HD groups respectively) (**Fig. 4.16**). It is evident that the expressions of TNF-α was decreased by 35.88% and 38.52% in LD and HD groups respectively when compared with FCA group animals; Cox-2 expression decreased by 31.65% and 34.96% in LD and HD respectively when compared with FCA group animals.



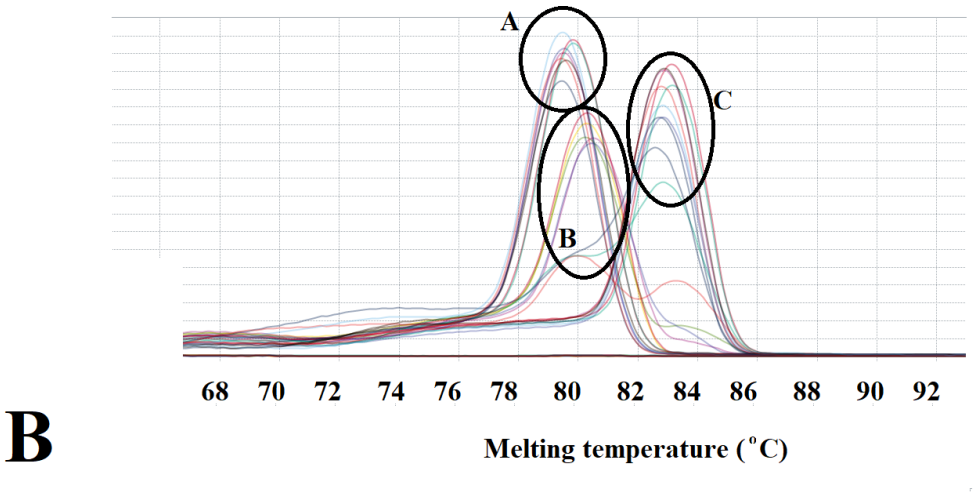


Figure 4.15. A. Amplification curves of all the three selected genes in reverse transcription quantitative PCR containing each experimental group (in duplicates) obtained from the Roche LightCycler 96 machine. Two negative control sets were used during the experimentation resulting in two different colours in the graph. **B.** the circle A represents dissociation temperature zone for TNF-α; Circle B represents dissociation temperature zone for GAPDH and circle C represents dissociation temperature zone for Cox-2 respectively.

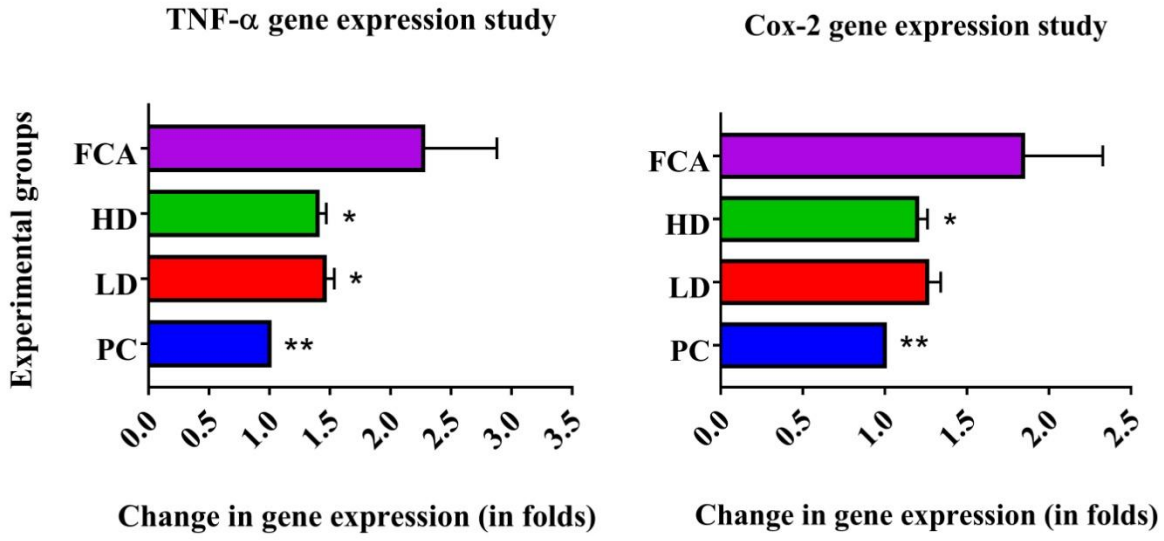


Figure 4.16. Relative expression changes in the Cox-2 and TNF-α genes assessed through reverse transcriptase real time quantitative PCR. The expression of fold-changes is expressed in bar diagram and the significance value of LD and HD groups are compared with FCA groups. PC refers to positive control; LD and HD are experimental groups. LD corresponds to 0.40 g of *Aloe vera* gel homogenate / kg b.w. and HD corresponds to 0.80 g of *Aloe vera* gel homogenate / kg b. w. FCA refers to arthritic control group. Significance value at $P \leq 0.05$ is indicated as *; Significance value at $P \leq 0.01$ is indicated as **.

Cytokines are the important mediators of inflammation initiation and progression. TNF-α is a pro-inflammatory cytokine released from the macrophages and monocytes at the initiation of inflammation and as the inflammation progresses, the other infiltrating immune cells also produce TNF-α. It also initiates the production of matrix metalloproteinases responsible for cartilage degradation (Saleem *et al*., 2019; Hassan *et al*., 2019). On the other hand, Cox-2, an immune modulator, is responsible for the increased production of prostaglandins which is responsible for increased pain and swelling at the site of inflammation (Sano, 2011). Cytokines along with Cox are target bio-molecules of the host body which have a significant role on inflammation progression. Non-steroidal anti-inflammatory drugs primarily function by inhibiting cycloxygenase pathway (Efthimiou and Kukar, 2010). Cytokine inhibitors have been introduced in the medication against inflammation as well.

The role of crude plant extracts on different cytokines, COX and other biomarkers during inflammatory arthritis has already been discussed in the section 2.5. Different ELISA

and real time PCR-based techniques have been used to explore the role of herbal extracts in the adjuvant induced arthritic animal models. *Moringa rivae* extract has shown down-regulation of TNF-α and prostaglandin E2 level at protein level assessment through ELISA (Saleem *et al.*, 2019). The up-regulation of pro-inflammatory cytokines, Cox-2 and other related genes like MCP-1, VEGF during the inflammatory arthritic condition has been investigated and explored by different researchers in Wistar rat models (Paquet *et al.*, 2012). Extracts of the plants like Fekugreek (*Trigonella foenum graecum*) (Suresh *et al.*, 2012), *Xanthium strumarium* (Lin *et al.*, 2014), *Trachyspermum ammi* (Korani and Jamshidi, 2020), *Withania somnifera* (Khan *et al.*, 2019), *Piptadeniastrum africanum* (Mbiantcha *et al.*, 2017), *Moringa rivae* leaf (Saleem *et al.*, 2019), etc has successfully down-regulated arrays of pro-inflammatory cytokines and biomarkers including Cox-2 in the model animal systems.

In this study, our result shows that the *Aloe vera* gel homogenate regulates TNF-α cytokine expression in experimental animals, thereby regulating the progression of inflammatory arthritis (**Fig. 4.15** and **4.16**). The regulation of gene expression is found to be in a dose-dependent manner, which is true for all the plant extracts. We previously discussed the possibility of Cox-2 inhibition by *Aloe vera* gel constituents through the results obtained from *in silico* docking (**Fig. 4.6**) and *in vivo* anti-inflammatory studies. Now it is evident that the gene expression of Cox-2 is interrupted by the *Aloe vera* gel constituents when fed orally in inflammatory arthritic conditions. Hence, the Cox-2 inhibition seems to be a very effective mode of action for the anti-inflammatory property of this plant.

4.5. COMPREHENSIVE DISCUSSION

The *Aloe vera* gel is a commonly used plant both as a food and as a traditional medicinal remedy (Ahlawat and Khatkar, 2011). The plant leaf gel is cooked or extracted into food products or consumed directly. In the ethnic consumption practices, the *Aloe vera* gel is generally consumed afresh and generally, mixture or extraction of the gel in alcoholic or other solvents is not well documented in ethnic practices. The present study, hence, was concentrated on the aqueous homogenized *Aloe vera* gel. A large amount of literature and evidences regarding the food use of this plant gives rise to a strong statement that the plant does not usually contain any strong toxic effect in the human body. Depending on these available information, the experimental plant sample preparation and dose-limit was designed. As discussed in the sections 2.3.4 and 3.2.2.1 of this dissertation, in ethnobotany and in Ayurveda, there are sufficient evidence regarding the usage of the plant gel in either crude unprocessed condition or in extracted condition ethnically (Morton 1961, Shedoeva *et al.*, 2019). This process has been noted during the preparation of the plant gel and the gel has been used in a crude unprocessed form which is directly homogenized in distilled water. In ethnic practices, the gel of this plant is either used topically or it is orally consumed freshly after collection without any extraction or processing.

In the present work, the gel was prepared fleshly everyday and no preservation process has been adopted. Along with that, the detailed study of the *Aloe vera* gel regarding its toxic effects has been done in this thesis. These findings serve as the basis for toxicological classification of *Aloe vera* gel. Acute toxicity tests determine the lethality value and indicate the maximum dose for the survival of test animals (Raza *et al.*, 2002). We determined that the orally fed highest dose of *Aloe vera* gel (5 g/kg b.w.) neither initiated any lethality nor showed harmful effects in experimental animals (**Table 4.1**). No obvious behavioral abnormalities were seen including body fur irritation, diarrhea or salivation. The *Aloe*-gel was therefore considered to be non-toxic and safe up to the dose of 5 g/kg b.w. and the lethality value was concluded as “unclassified” up to the feeding range as per guideline. On the other hand, sub-chronic toxicity study is widely used tool to assess the harmful effects associated with long-term repeated exposure of the animals to a compound, drug or formulation and provides detailed information about the organ-based toxicity (National

Toxicology Program, 2001). The oral consumption of *Aloe vera* gel for a period of 28 days did not initiate any sickness symptoms or death issues, did not alter the body weight of the gel fed rats (**Fig. 4.1**). The histological (**Fig. 4.2**), serum enzymatic and biochemical parameters (**Fig. 4.3**) also remained unaltered. The orally consumable doses of *Aloe vera* gel to be used for extended period of time (28 days for anti-inflammatory and anti-arthritic tests) were determined following the OECD guidelines (OECD 407, adopted on October 3, 2008). The selection of orally consumable doses of *Aloe vera* gel and its preparation was justified considering the background studies.

The extensive experimentation leading to the exploration of the toxic properties of *Aloe vera* gel provided enough evidences to conclude that the Aloe gel homogenate had no harmful side effects in the model system and thus in human body. The *in silico*, *in vitro* and *in vivo* toxicological approaches have led to the usage of the plant gel homogenate as an anti-inflammatory and anti-arthritic remedy. From the *in vitro* and *in vivo* anti-inflammatory tests, it was evident that the plant gel has substantial role in the amelioration of inflammation by stabilizing lysosomal membrane lysis (**Fig. 4.8**), by inhibiting protein denaturation (**Fig. 4.8**) and by inhibiting cyclooxygenases (Cox) expression as well (**Figs. 4.6; 4.15**). The NSAID-like role of the plant homogenate by hindering Cox activity (Selvam and Jachak, 2004) is supported by the current work as the model inflammatory arthritic animals showed down-regulation of Cox-2 gene expression experimentally, when orally treated with *Aloe vera* gel (**Figs. 4.15; 4.16**). As one of the most important cytokines, TNF-α plays a major role in the inflammatory conditions (section 2.2.4). As seen from the real time PCR-based gene expression studies in inflammatory models, it was postulated that there is a high chance of interaction between the plant gel phyto-constituents and prime cytokine like TNF-α genes (**Figs. 4.15** and **4.16**) resulting in the down-regulating the elevated expression of TNF-α in inflamed condition.

The rich medicinal properties of this plant have been used to cure diabetes in experimental animals (Luka and Tijjani, 2013), as an would healer (Davis *et al*., 1994), as a skin medication (Surjushe *et al*., 2008), and in inflammatory diseases. The *Aloe vera* gel has Aloe-emodin (*anthraquinone*), acemannan (mesoglycan) and many more bioactive compounds as its major constituent (Kshirsagar *et al.,* 2014; Shedoeva *et al*., 2019). In the present work, the report on the ameliorative role of unprocessed *Aloe vera* gel homogenate in

the regulation of inflammatory and arthritic symptoms in experimental rats has been explored and discussed. The paw-circumference, serum biochemical parameters and blood profile of the inflammatory arthritic rats were brought back to normal levels after oral feeding of the plant gel homogenate (**Figs. 4.11** to **4.14**). The study also shows that the *Aloe vera* gel homogenate concomitantly down-regulates both TNF-α and Cox-2 in the experimental animal groups (**Fig. 4.16**).

This report can be considered as a baseline data describing the efficacy of crude unprocessed plant product against the inflammatory arthritis. The crude unprocessed homogenized form of *Aloe vera* leaf gel has been orally administered, a method which is in traditional use by different ethnic communities of Egypt, Rome, Africa and Asia (Shedoeva *et al.*, 2019). By using unprocessed *Aloe vera* gel, we presume, the natural constituents of the gel were restored in its natural proportion and thus natural synergistic role of the gel had been monitored. The *Aloe vera* gel looks like a promising complementary medication against inflammation and associated pain in chronic inflammatory conditions such as in RA.