ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Effects of Vitamin-E, Morin, Rutin, Quercetin against Doxorubicin in Rabbits: A Hematological Study

Raja Kumar Parabathina*[1], Muralinath E [2], Lakshmana Swamy P [3], Hari Krishna VVSN [3], Shanthi Sree K [3].

[1]Department of Biochemistry, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur-522510.Andhra Pradesh, India.
[2]Department of Physiology, NTR College of Veterinary Science, Gannavaram-521101.Andhra Pradesh, India.
[3]Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur-522510.Andhra Pradesh, India.

ABSTRACT

Vitamin-E and flavonoids are natural anti-oxidants present in the vegetable diet. The present study was conducted on New Zealand white rabbits aged between 3-6 months and averaging 1.5-3.0 kg in weight. Rabbits are divided into 5 groups of 6 in each; a 4-week feeding trail is used to evaluate the hematology of rabbits by feeding vitamin-E, flavonoids morin, rutin, and quercetin along with drug doxorubicin. Doxorubicin is a potential chemotherapeutic agent for the treatment of a variety of human malignancies. In the present study, the development of cardio toxicity was prevented by reducing oxidative stress using natural antioxidant vitamin E (50 IU/kg body weight) and flavonoids morin, rutin and quercetin. The four weeks treatment of flavonoids (20mg/kg body weight) were affectively controlled the oxidative stress induced cardiomyopathy in rabbits by doxorubicin (10mg/kg body weight) treatment for two days. The flavonoids regulated the hematological parameters at optimum levels. The hematological components of study included red blood cells (RBC), white blood cells WBC, Hemoglobin (Hb), lymphocytes (LYM), platelets (PLT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), basophiles (BAS), eosinophiles (EOI), monocytes (MON), and neutrophiles (NEU). Results showed that hematological parameters were affected ($P<0.05$) by experimental diets. The overall conclusion of this study is that the flavonoids can act as antioxidants and they can control the alteration in the hematological parameters. By this study, the authors suggests that the flavonoids are natural anti-oxidants, which can be used in the various treatments of cancers as supplement to reduce the oxidative stress produced by the drugs.

Key words: oxidative stress, cardiomyopathy, doxorubicin (DOX), morin, rutin, quercetin.

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Food is the major sources of antioxidants like vitamin C, vitamin E, selenium, and carotenoids that may help in providing protection against diseases by contributing, along with enzymes involved in scavenging of free radicals, to the total antioxidant defense system of the human body. Flavonoids are polyphenolic compounds present in the many plant derived foods. Many epidemiological studies have shown that flavonoid intake is inversely related to mortality from coronary heart disease and to the incidence of heart attacks. Recent studies have demonstrated that flavonoids found in fruits and vegetables may also act as antioxidants. Like vitamin-E, flavonoids contain chemical structural elements that may be responsible for their antioxidant activities. Blood is a complex fluid of various cells and tissues, containing large variety of dissolved suspended inorganic and organic substances [1] or specialized circulating tissues and cells suspended in the intercellular fluid substances [2] which circulates in the arteries, vessels and capillaries of man and animals [3] and whose primary function is to transport oxygen from respiratory organs to body cells [4] distributing nutrients and enzymes to cells and carrying away waste products [5] thereby maintaining homeostasis of the internal environment [6]. The various functions of the blood are made possible by the individual influenced and collective actions of its constituents- the hematological and biochemical components. The biochemical and hematological components are influenced by the quantity and quality of feed and also the level of anti-nutritional elements or factors present in the feed [7]. Hematological components of blood are also valuable in monitoring feed toxicity especially with feed constituents that affect the formation of blood [8].

Doxorubicin is a potent chemotherapeutic agent for the treatment of a variety of malignancies and doxorubicin (DOX) in one of the most widely used broad-spectrum anti cancer agents. The dose related cardiomyopathy and congestive heart failure due to doxorubicin has limited the use of this drug. Cardiovascular diseases (coronary artery disease, hypertension, heart failure, and stroke) are the leading causes of death in human beings of modern days. Oxidative stress is the unifying mechanism for many cardiovascular risk factors (diabetes and obesity) [9].

The capacity of flavonoids to act as antioxidants depends upon their molecular structure. Morin, rutin and quercetin by acting as antioxidants exhibited several beneficial effects, such as anti-inflammatory, antiallergic, antiviral as well as an anticancer activity. Quercetin, the most abundant dietary flavonol, is a potent antioxidant because it has all the right structural features for free radical scavenging activity. It is evident that the flavonoids play an important role in the various types of metabolic activities of life. They have also been suggested to play a protective role in liver diseases, cataracts, and cardiovascular diseases. According to their specificity in antioxidation function, certain selected flavonoids naturally available in diet (morin, rutin and quercetin) are used in the present study to investigate their affects on hematological studies doxorubicin induced oxidative stress in rabbits.

ISSN: 0975-8585

Keeping in view the present study was aimed to estimate the protective effect of different flavonoids (morin, rutin, and quercetin) against treatment of doxorubicin on hematological changes in rabbits.

MATERIALS AND METHODS

Experimental site:

Thirty apparently healthy, New Zealand white rabbits weighing 2.5 to 3.0 kg (about 3-6 months) were obtained from Laboratory of small animal house, Department of Pharmacology, Dr.Pinnamaneni Siddhartha Institute of Medical Sciences and Research Foundation, Chinnoutapalli, Gannavaram, Krishna District, Andhra Pradesh, India. The animals were housed in the cages of departmental laboratory animal shed. All the animals were fed with control diet during a month acclimatization period. All animals were kept under uniform managerial and standard hygienic conditions through the experimental period. All the rabbits were weighed and randomly housed, two animals in each cage. The cages were located in a well ventilated house and all the animals had free access to feed and water at all times.

Experimental Design:

Thirty rabbits were randomly divided into five groups and six animals in each group. The group I animals are Controls which were fed normal diet about twenty eight days and the twenty ninth and thirtieth day i.e. two days doxorubicin 10 mg /kg body weight was given intra venously, for the group II animals antioxidant vitamin E 50 IU/ kg body weight was given orally along with normal diet for twenty eight days and the two doses of doxorubicin was administrated on twenty ninth and thirtieth day, for the group III, IV & V were fed orally with flavonoids morin, rutin and quercetin, 20mg/ kg body weight along with *ad libitum* of water and diet respectively for twenty eight days and the doxorubicin was administrated in two doses on twenty ninth and thirtieth day as in group I and II rabbits. At weekly intervals blood samples were collected and analyzed foe hematological parameters. On 5^{th} time i.e. after doxorubicin treatment for two days, again blood samples were collected and analyzed for hematological parameters.

Methods:

Hematological study of all parameters was carried out as per standard methods described by Jain [10]. Total leukocyte count was carried out by hemocytometer. For differential leukocyte count, alcohol fixed blood smears were stained with Giemsa's stain. Hemoglobin concentration was measured calorimetrically according to the method described by Richterich [11]. Ammonium Hydroxide: 4 ml of ammonium hydroxide (specific gravity 0.91, about 25% purity) was added to 996 ml of distilled water and stored in brown bottle. Normality of the solution was 0.007N. Five ml of 0.007N ammonium hydroxide was taken in a test tube.

ISSN: 0975-8585

Then 0.02 ml of packed RBC was added and mix well. The colour developed was stable for few hours. Optical density was measured at 578 nm against 0.007N ammonium hydroxide as blank.

g of Hb /100ml = Absorbance at 578 nm x 26.3

From the estimated values of hemoglobin (g/dl), RBC count (millions/mm) and PCV (volumes %), the following indices can be calculated. [12].

Mean corpuscular volume (MCV),

$$\text{MCV in } mm^3 = \frac{\text{PCV} \quad \text{X } 10}{\text{RBC count}}$$

Mean corpuscular hemoglobin (MCH),

$$\text{MCH in pg} = \frac{\text{Hemoglobin} \quad \text{X } 10}{\text{RBC count}}$$

Mean corpuscular hemoglobin concentration (MCHC),

$$\text{MCHC \%} = \frac{\text{Hemoglobin} \quad \text{X } 10}{\text{PCV}}$$

All data were subjected to analysis of variance (ANOVA) applicable to a completely randomized design [13]. Significant means were separated using Duncan's multiple range test [14]

RESULTS AND DISCUSSION

Table I reveals the effect of experimental diet on RBC, WBC, Hemoglobin and Lymphocytes concentration on doxorubicin treatment. The RBC count obtained in all the groups of rabbits at the end of 4th week were apparently in the normal range i.e. 3.97±0.38 to 4.66±0.07 X 10^6/uL except in quercetin treated group (2.42±0.32 X 10^6/uL). The values obtained in the present study for RBC were in agreement with previous studies conducted in rabbits [15-17]. Treatment of doxorubicin in rabbits for 2 days at the end of 4 weeks of group I and group III resulted a significant decrease in RBC count from 4.18±0.32 to 2.86±0.31 X 10^6/uL and 4.36±0.27 to 2.86±0.37 X 10^6/uL respectively. The RBC count in vitamin E, rutin and quercetin treated rabbits was the almost same RBC even after the treatment of doxorubicin. Similarly, the treatment of anthracycline antibiotic daunorubicin in rabbits results a significant decrease in RBC count [18]. It is well established that the anthracycline antibiotics like daunorubicin and doxorubicin were myelosupressents and thereby affecting erythropoiesis. It is evident from the

results obtained from the present study that the flavonoids rutin, quercetin and vitamin E could impede the down regulation of erythropoiesis induced by doxorubicin in rabbits.

The WBC count in rabbits was in normal range throughout the study (4 weeks) i.e. from 6.50±0.89 to 8.30±0.15X 10^3 /uL in all groups (I-V). After the DOX treatment for 2 days the WBC count was significantly decreased in group I from 7.90±0.60 to 3.91±0.52 X10^3 /uL. Rutin treated rabbits maintained the normal WBC levels even after the treatment of doxorubicin. However, there was a significant decrease in WBC count in the morin and quercetin treated animals. Similarly, it was reported that treatment of anthracycline antibiotic daunorubicin in rabbits resulted a significant decrease in WBC count [18].

The normal range of the hemoglobin from the previous studies made by Kaneko, J.J, (1989) [19] was 9.4 to 17.4 g/dL. In the present study, the hemoglobin levels were in the range of 6.80±0.26 to 7.34±0.39 g/dL in the period of 4 weeks. The control group after treatment of doxorubicin for 2 days decreased the Hemoglobin from 7.34±0.39 to 6.73±0.53 g/dL. Vitamin E, morin, rutin and quercetin treated rabbits could maintain the almost same hemoglobin levels even after the doxorubicin treatment. That indicates, the flavonoids could maintain the hemoglobin levels even in the DOX-induced cardiomyopathy. On anthracycline treatment also the hemoglobin levels were decreased significantly at the end of the experiment [18].

Table: 1- Hematological parameters: RBC, WBC, Hemoglobin and Lymphocytes concentration in Mean±SEM on treatment of flavonoids with doxorubicin

Parameters	RBC		WBC		Hemoglobin		Lymphocytes	
Groups	28 days treatment	Doxorubicin treatment	28 days treatment	Doxorubicin treatment	28 days treatment	Doxorubicin treatment	28 days treatment	Doxorubicin treatment
Control	4.18±0.32	2.86±0.31	7.9±0.60	3.91±0.52	7.34±0.39	6.73±0.53	5.77±0.79	3.61±0.27
Vit-E	3.97±0.38	2.74±0.27	7.5±0.36	5.91±0.49	6.8±0.42	6.95±0.64	5.23±0.67	3.71±0.59
Morin	4.36±0.27	2.86±0.37	8.3±0.15	5.63±0.24	7.16±0.40	6.88±0.80	2.96±1.12	5.2±0.51
Rutin	4.66±0.07	4.14±0.43*	7.35±0.75	7.68±0.95**	6.8±0.26	7.45±0.78	3.22±1.11	3.87±0.89
Quercetin	2.42±0.32**	2.16±0.18	6.5±0.89	4.88±0.48	5.56±0.88	5.96±0.54	5.37±0.78	3.66±0.84

* In a row differ significantly at P<0.05, ** In a row differ significantly at P< 0.01

In this present study, lymphocytes were in the range of 2.96±1.12 to 5.77±0.79 X10^9/L, in control group after the treatment of doxorubicin for 2 days at the end of 4 weeks of normal diet, doxorubicin decreased the lymphocytes from 5.77±0.79 to 3.61±0.27 X10^9/L. Vitamin E and quercetin treated rabbits were also decreased the lymphocytes, where as morin treated rabbits increased the lymphocytes and rutin maintained the lymphocytes even after the treatment of doxorubicin. In the experimental animals the lymphocyte count was in the range of 3 - 9 X10^9/L [16]. So, the levels were maintained even after the DOX treatment.

ISSN: 0975-8585

The MCV levels in normal rabbits was 50-75 mm^3 [16], where as in control group of this study was maintain the MCV levels even after the DOX treatment for 2 days, from 65.55±1.00 to 65.45±1.97 mm^3 (table II). Vitamin E treated rabbits were also maintained the same as controls, where as morin treated group increased the MCV from 62.33±1.13 to 67.04±0.82 mm^3. The rutin treated group were decreased the MCV from 68.56±1.86 to 64.02±1.28 mm^3 and quercetin treated rabbits increased a little in the MCV after the treatment of doxorubicin. This shows that the DOX did not affect the MCV levels.

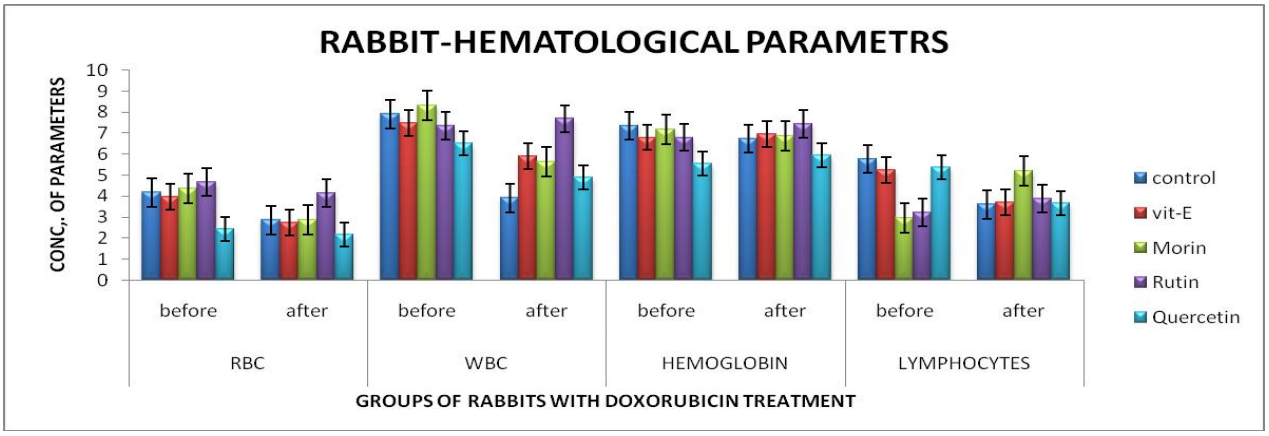

Fig: 1- Hematological parameters: RBC, WBC, Hemoglobin and Lymphocytes concentration in Mean±SEM on treatment of flavonoids with doxorubicin. (Before: Upto 4th week value, after: After the treatment of Doxorubicin)

The range obtained from the previous studies done by [16] on experimental rabbits MCH was 18-24 pg/cell. But the present study had shown the levels as 25.73±1.28 to 32.77±2.05 pg/cell. The control group after the treatment of doxorubicin for 2 days at the end of 4 weeks of normal diet, the decreased MCH levels from 31.05±1.43) to 24.46±0.30 pg/cell were found. Vitamin E and morin treated animals were maintained the MCH levels even after the treatment of doxorubicin, where as rutin treated animals were decreased the levels from 32.77±2.05 to 27.15±2.26 pg/Cell. In quercetin treated rabbits were decreased the MCH levels from 29.29±1.68 to 27.45±2.04 pg/Cell was very little change. This indicates that DOX treatment had no significant affect on the MCH.

Table: 2- Hematological parameters MCV, MCH, MCHC concentration in Mean±SEM on treatment of flavonoids with doxorubicin

Parameters	MCV		MCH		MCHC	
Groups	28 days treatment	Doxorubicin treatment	28 days treatment	Doxorubicin treatment	28 days treatment	Doxorubicin treatment
Control	65.55±1.00	65.45±1.97	31.05±1.43	24.46±0.30	52.98±3.81	35.79±2.08
Vit-E	65.3±1.81	65.03±0.82	25.73±1.28	25.89±0.61	42.18±3.74	40.53±1.39
Morin	62.33±1.13	67.04±2.58	27.43±1.69	25.43±1.28	45.9±3.27	38.66±1.64
Rutin	68.56±1.86	64.02±1.28	32.77±2.05	27.15±2.46	46.35±4.88	41.46±4.37
Quercetin	63.01±1.41	64.65±1.39	29.31±1.68	27.45±2.04	46.8±2.43	39.08±1.09

* In a row differ significantly at P<0.05, ** In a row differ significantly at P< 0.01

ISSN: 0975-8585

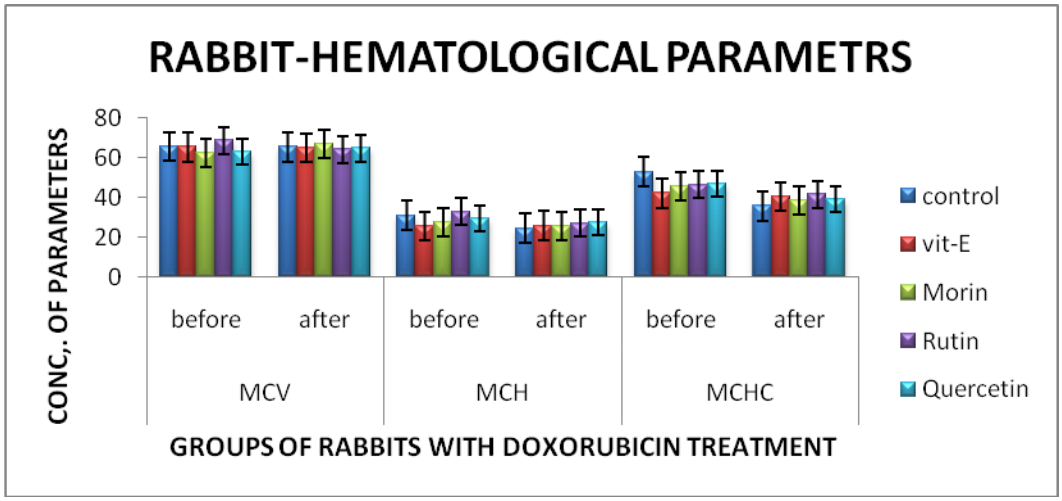

Fig: 2- Hematological parameters MCV, MCH, MCHC concentration in Mean±SEM on treatment of flavonoids with doxorubicin. (Before: Upto 4th week value, after: After the treatment of Doxorubicin)

In the present study, a significant decrease of MCHC levels were observed in the control group after the treatment of doxorubicin for 2 days at the end of 4 weeks treatment of normal diet, from 52.98±3.81 to 35.79±2.08%. Vitamin E treated group were maintained the MCHC levels, where as morin, rutin and quercetin treated rabbits were also decreased the MCHC. The normal range was 27 to 34 [16], but this study shown that the MCHC levels were more than the normal range. So, it gives a clarification that there was no significant affect was noticed on DOX treatment.

Table: 3- Hematological parameters: Platelets concentration in Mean±SEM on treatment of flavonoids with doxorubicin

Parameter	Platelet	
Groups	28 days treatment	Doxorubicin treatment
Control	118.5±2.51	92.16±6.42
Vit-E	172±3.74**	149.83±2.99**
Morin	131.5±2.46*	139.5±4.50**
Rutin	141.83±4.07**	241±3.97**
Quercetin	124.16±3.45	127.33±2.84**

* In a row differ significantly at P<0.05, ** In a row differ significantly at P< 0.01

ISSN: 0975-8585

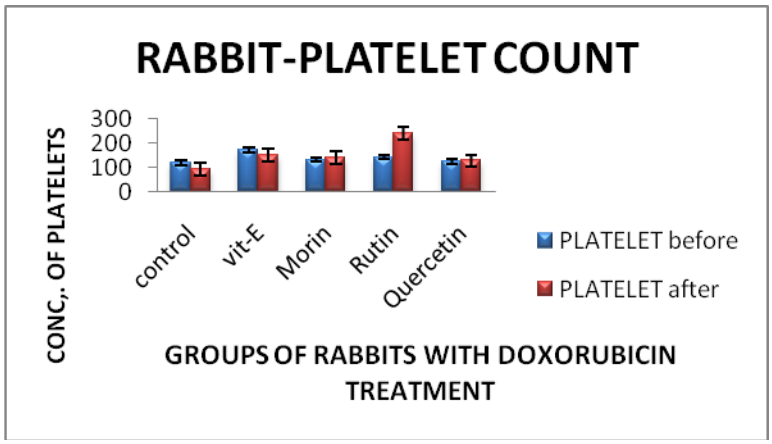

Fig: 3- Hematological parameters: Platelets concentration in Mean±SEM on treatment of flavonoids with doxorubicin. (Before: Upto 4th week value, after: After the treatment of Doxorubicin)

The normal range of platelets in rabbits was up to 290 X 10^3/mm^3 [16]. In the present study the range of platelets was 118.50±2.51 to 172.00±14.98 X 10^3/mm^3 (table III). The control group after the treatment of doxorubicin for 2 days at the end of 4 weeks of normal diet, doxorubicin decreased the platelets from 118.50±2.5 to 92.16±6.42 X 10^3/mm^3. Vitamin E treated rabbits were also decreased the platelets, but morin, rutin and quercetin treated rabbits increased the platelets. So, the flavonoids were able to protect the platelet count in the oxidative stress induced by DOX.

The basophils range was 0.19±0.01 to 0.23±0.04 X10^9/L in this study, but the normal range in previous studies was 0 - 0.84 [16]. In control group after the treatment of doxorubicin for 2 days at the end of 4 weeks of normal diet, doxorubicin decreased basophils from 0.23±0.04 to 0.21±0.05 X10^9/L. Vitamin E and quercetin treated rabbits were increased the basophils, where as morin and rutin maintained the basophiles even after the treatment of doxorubicin (table IV).

Table: 4- Hematological parameters: Basophiles, Eosinophiles, Monocytes and Neutrophiles concentration in Mean±SEM on treatment of flavonoids with doxorubicin

Parameters	Basophiles		Eosinophiles		Monocytes		Neutrophiles	
Groups	28 days treatment	Doxorubicin treatment	28 days treatment	Doxorubicin treatment	28 days treatment	Doxorubicin treatment	28 days treatment	Doxorubicin treatment
Control	0.23±0.04	0.21±0.05	0.33±0.07	0.19±0.03	0.21±0.01	0.15±0.02	0.14±0.06	0.15±0.01
Vit-E	0.19±0.01**	0.22±0.05	0.31±0.05*	0.17±0.02*	0.16±0.01**	0.14±0.06	0.13±0.02	0.14±0.06
Morin	0.2±0.05**	0.2±0.05	0.35±0.06*	0.22±0.04**	0.15±0.05**	0.13±0.01**	0.14±0.06	0.15±0.09
Rutin	0.23±0.04	0.22±0.05	0.35±0.05*	0.38±0.05**	0.14±0.01**	0.12±0.01**	0.15±0.06	0.13±0.02*
Quercetin	0.2±0.05**	0.23±0.04*	0.5±0.10**	0.33±0.07**	0.14±0.06**	0.12±0.05**	0.16±0.01*	0.16±0.01

* In a row differ significantly at P<0.05, ** In a row differ significantly at P< 0.01

ISSN: 0975-8585

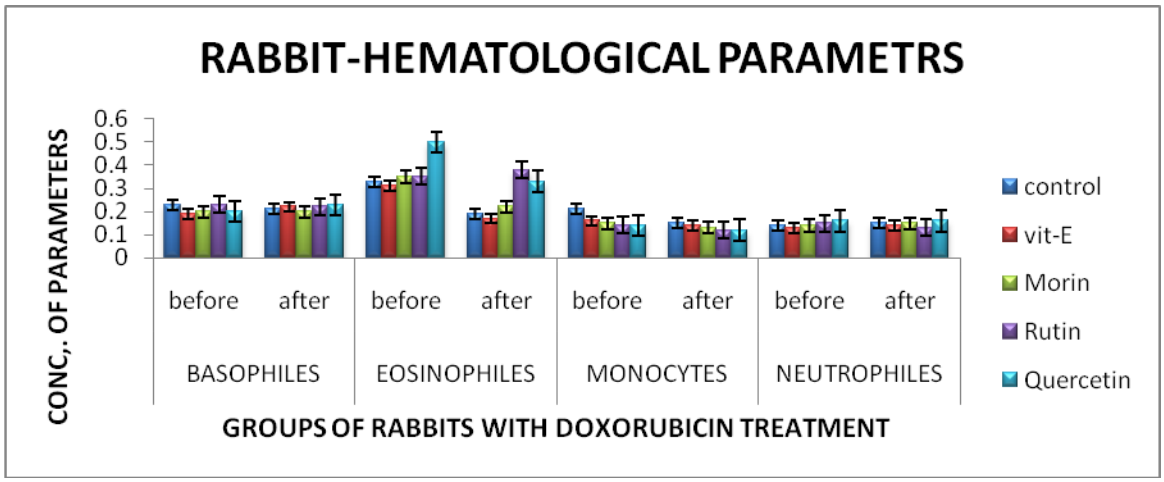

Fig: 4- Hematological parameters: Basophils, Eosinophils, Monocytes and Neutrophils concentration in Mean±SEM on treatment of flavonoids with doxorubicin. (Before: Upto 4th week value, after: After the treatment of Doxorubicin)

The Eosinophiles range of the rabbits was 0 - 2 [16] or 1.0 - 2.5% [20]. But in our study the levels were 0.31±0.05 to 0.50±0.10 X10^9/L. In control group after the treatment of doxorubicin for 2 days at the end of 4 weeks of normal diet, doxorubicin decreased the eosinophiles from 0.33±0.07 to 0.19±0.03 X10^9/L. Vitamin E, morin and quercetin treated rabbits were also decreased the eosinophiles, where as rutin treated rabbits increase very little of the eosinophils even after the treatment of doxorubicin. Finally the eosinophil count was maintained even after the treatment of DOX. From the present study the monocytes were in the range of 0.14±0.01 to 0.21±0.01 X10^9/L, but the previous studies had shown the value as <0.5 X10^9/L [16]. In control group after the treatment of doxorubicin for two days at the end of 4 weeks of normal diet, doxorubicin decreased the monocytes from 0.21±0.01 to 0.15±0.02 X10^9/L. Vitamin E, morin, rutin and quercetin treated rabbits were maintained almost normal even after the treatment of doxorubicin.

The present study showed that the neutrophils were in the range of 0.13±0.02 to 0.16±0.01 X10^9/L, in control group after the treatment of doxorubicin for 2 days at the end of 4 weeks of normal diet, doxorubicin maintained from 0.14±0.06 to 0.15±0.01. Vitamin E, morin, rutin and quercetin treated groups were also maintained normal even after the treatment of doxorubicin. From the previous studies [16], the range of neutrophils was 0 - 0.2, compared to this value, the present studied values were maintaining the normal range even after the DOX treatment.

CONCLUSION

The present study concludes that the flavonoids are natural anti-oxidants available in natural vegetable diet. The rabbits of group III, IV and V which were provided the flavonoids (morin, rutin and quercetin) along with diet showed remarkable changes indicating the maintenance of hematological parameters on the treatment of doxorubicin. The morin, rutin

and quercetin can protect the doxorubicin induced oxidative stress by regulating the changes in hematological parameters of the rabbits. Dietary intervention of flavonoids may be a good practice to protect myocardium in the doxorubicin treatment of cancers or malignancies. Flavonoids like quercetin, rutin and morin could be good adjunct molecules in doxorubicin therapy. Further investigations are necessary to prove clinical efficacy and use of flavonoids in mitigation of doxorubicin oxidative stress leading to cardiomyopathy. Thus, this study had an advantage to conduct experiments in the rabbits as a model in the research of Biochemistry, Physiology and Pharmacology etc.

REFERENCES

[1] Stewart M. Animal Physiology. The Open University U.S.A. 1991;132-133.
[2] Dellman H and Brown E. Text book of Veterinary Histology. LEA and Febilger Philadelphia. 1976: 88-96.
[3] Kronfield OW and Mediway NC. Blood Chemistry In: Text book of Veterinary Clinical Pathology. Williams and Williams Co. Baltimore 1975: 81-96.
[4] Duke HH.Duke's Physiology of Domestic Animals. 1975; 8:33.
[5] Baker FS and Silverton RE. Introduction to medical laboratory technology, Butterworth S.C London, 1982; 5:481-494.
[6] Bentrick S. Hematology, Textbook of Veterinary Pathology. Williams and Williams Co. Baltimore, 1974: 217-224.
[7] Akinmutimi AH. Evaluation of sword bean (Canavalia gladiata) as an alternative feed resources for broiler chickens. Ph.D Theses. Michael Okpara University of Agriculture, Umudike, Nigeria 2004.
[8] Oyawoye EO and Ogunkunle M. Proc Nig Soc Anim Prod 1998; 23:141-142.
[9] Leila Bettina Seres. "Oxidative stress in cardio vascular diseases and in experimental models" PhD thesis. 2006: 74.
[10] Jain NC. Hematological techniques In: Schalm's Veterinary Haematology, Lea and Febiger, Philadelphia. 1986; 4:20-50.
[11] Richterich R. Clinical chemistry theory and practice in "S.Karger, Baser (Switzerland)" Acad press. NY and London 1969: 336-337.
[12] Raghuramulu N, Madhavan Nair K, Kalyanasundaram S. A manual of laboratory techniques. 2003; 2: 309.
[13] Myers CE, Mc Guine WP, Liss RH, Ifrim I, Young RC. "The role of lipid peroxidation in cardiac toxicity and tumour responce", Science, 1977; 197: 165-167.
[14] Santos AC, Vyemura SA, Lopes JL, Bazon JN, Mingotto FE, Cutric. Free Radic Biol Med 1998; 24: 1455-61.
[15] Jones RT. Lab Anim 1975; 9(2):143-7.
[16] Mitruka BM, Ranwnley HM. Clinical Biochemical and hematological reference values in normal and experimental animals. Masson publishing USA, 1977; 83:134- 5.
[17] Burke J. "Clinical care and medicine of pet rabbit, proceedings of the Michigan" Veterinary Conference 1994: 49-77.

[18] Tomas Simunek, Ivona Klimtova, Jana Kaplanova, Y vona Mazurova, Michaela Adamcova, Martin Sterba, Radomir Hrdina, and Vladimer Gersl. Euro J Heart Failure 2004; 6: 377-387.
[19] Kaneko J J. "Clinical biochemistry of domestic animals", Academic Press, New York 1989.
[20] Ahamefule F O, Eduok G O, Usman A Amaefule K U, Obua B E & Oguike S A. Pak J Nut 2006; 5(3): 248-253.