

Effect of Quercetin on Cyclophosphamide Induced Biochemical Profiles in Rat Liver

*¹Aamir Javed, ¹Ashwini L.S

¹Base Fertility Medical Science Pvt Ltd,
KHB Colony, Basaveshwarnagar,
Bangalore, India

*aamir.javed0077@gmail.com

²Muralidhar T.S

²Dept of Biotechnology, R&D Center,
Dayananda Sagar College of Biological
Sciences,

Kumaraswamy Layout, Bangalore, India.

³Sneh Sagar

³Genelon Institute of Life Sciences Pvt Ltd.
No. 1160, First Floor,

Above State Bank of Mysore,
Government Hospital Road, Yelahanka Old
Town, Bangalore, India

⁴Siva Kumar Medam

⁴St Jhon Research Institute, Dept of
Microbiology,
Arjapur Road, Koramangala 2 Block,
Madiwala, Bengaluru, Karnataka, India

Abstract: *Stress is known to affect synaptic plasticity; dendritic morphology and induces neurotoxic damage in humans, probably through generation of free radicals. In the present study, mice were selected and stress was induced using cyclophosphamide. For this it was given 1ml dose (40 mg/kg/day) for fifteen days. Another mouse was given anti-stress dose of quercetin (250mg/kg/day). It was treated with 1ml dose for thirteen days and on last two day it was given Cyclophosphamide to induce stress. A normal mouse was also kept in same condition as other two mice except that it was not stressed as was used as standard reference in the present study. At the end of one week the all the three mouse were sacrificed and liver was obtained for research. CYP is an alkylating agent which has as carrier a phosphorylated ring. Cyclophosphamide has various toxic effects, which include alopecia, nausea, vomiting, leukopenia and to a lesser degree thrombocytopenia, mucous ulcerations. However quercetin is a flavanoids of family of phenolics compounds widely found in the cabbage, leek, and broccoli. It has got the free radical scavenging activity. Quercetin supplementation improves running time to fatigue by stimulating mitochondrial biogenesis in mice. The Biochemical changes occurred in the mouse was observed by studying total nucleic acid, LPO, GSH, MDA, SOD and protein content changes in the treated mouse liver, and normal mouse was used as a reference. Estimation of nucleic acid was also performed by gel analysis. All readings were taken in triplicate to get rid of any errors.*

Keywords: *Cyclophosphamide, Quercetin, Mutation induction, Biochemical, Nucleic acid, Free radicals & Stress tolerance.*

1. INTRODUCTION

Flavanoids are a large family of phenolics compounds or polyphenols with wide therapeutic applications (Alexander *et al* 2006). Quercetin is one of the most widely spread naturally occurring flavanoids, found in onions, garlic, cabbage, leek, broccoli, apples, blueberries, tea and red wine. It is known that quercetin may exhibit anti-oxidant properties due to its chemical structure, particularly the presence and location of the hydroxyl (-OH) substitutions. Despite the fact that after long-term intake there is a wide distribution of quercetin (including its metabolites) in all tissues; toxic effects have not been reported until the dose reached 157 mg per kg/day. Quercetin is a natural polyphenolic compound currently under study for its ergogenic capacity to improve mitochondrial biogenesis. Quercetin might improve endurance performance since it is known that some polyphenols like quercetin and resveratrol improve aerobic capacity of skeletal muscle by promoting mitochondrial biogenesis in mice (Azuma *et al* 2010). Quercetin supplementation improves running time to fatigue by stimulating mitochondrial biogenesis in mice (Bowman *et al* 1983). Quercetin was shown to increase blood lactate production after high-intensity exercise. A psycho stimulant effect of quercetin has also been reported in vitro in a

manner similar to that of caffeine, but this effect was not found in human subjects. Quercetin has also been shown to reduce illness after strenuous exercise (Cheuvront *et al* 2009). In a mice model (De Boer *et al* 2005). However, these anti-inflammatory effects seen in vivo are not as powerful as those previously described in vitro. The differences are even greater when the in vivo data is obtained from athletes over half of the mature hepatocytes in mice and humans are aneuploid and yet retain full ability to undergo mitosis. This observation has raised the question of whether this unusual somatic genetic variation evolved as an adaptive mechanism in response to hepatic injury (Cupps TR *et al* 1982, Nishimura *et al* 1986). However Cyclophosphamide (CPA) is an alkylating agent which has as carrier a phosphorylated ring which must be released in the liver through the action of oxidase and phosphoramidase in order for the drug to act. The exact mechanism through which cyclophosphamide exerts its effect is not known, but most authors agree that it affects the G2- and S-phases of the cell-cycle (Davis *et al* 2008). Cyclophosphamide has various toxic effects, which include alopecia, nausea, vomiting, leukopenia and to a lesser degree thrombocytopenia, mucous ulcerations, brief spells of dizziness, transverse striations in the fingernails, increased skin pigmentation, pulmonary fibrosis, liver poisoning and facial abrasions (Ferré S *et al* 2008). Cyclophosphamide, administered either alone or in conjunction with other drugs, has proved an effective treatment in different types of tumor (disseminated Hodgkin's disease and non-Hodgkin lymphomas, acute leukemia, cancer of the breast and ovaries, myeloma, etc. Cyclophosphamide is a frequently used drug in anticancer chemotherapy (Middleton *et al* 2000). This presents a form of inactive transport, its metabolism and activation being located at the liver level under the action of microsomal enzymes releasing chemical and biological active compounds (Hardwood *et al* 2007). Thus, the effect of this cytostatic is strictly dependent on liver's functional status, its modification contributing significantly to diminish antitumoral action of cyclophosphamide. Under anaerobic conditions, in comparison to liver microsomes obtained from normal controls, liver microsomes obtained from rats pretreated with cyclophosphamide formed significantly less 7-deoxydoxorubicinol aglycone (P less than or equal to .05), whereas the disappearance of doxorubicin and the formation of 7-deoxydoxorubicin aglycone were unaffected (Manach C *et al* 2004). When directly investigated, the reduction of 7-deoxydoxorubicin aglycone to 7-deoxydoxorubicinol aglycone by microsomes was inhibited by cyclophosphamide pretreatment. the existence of a microsomal carbonyl reduction system for anthracycline antibiotics and indicate that cyclophosphamide does affect the metabolism of doxorubicin; in rats, this interaction results only in an alteration of the relative concentrations of presumably inactive metabolites, the 7-deoxyaglycones (Lagouge M *et al* 2006, Nieman *et al* 2007).

2. MATERIAL AND METHODS: METHODOLOGY DISCUSSED

2.1. Animals

Adult male Swiss albino mice weighing 25–30 g (10–12 weeks old) were obtained from the Central Animal facility, Sri Raghavendra Enterprises. Bangalore. India (Reg. no. 967/01/13/CPCEA), were used for the study. The animals were maintained under standard conditions of humidity, temperature ($25 \pm 2^\circ\text{C}$), and light (12-h light/12-h dark). They were fed with a standard mice pellet diet and had free access to water (Sener *et al* 2003, Su j. F *et al* 2003 & Watsonet *et al* 1985).

2.2. Drugs and Chemicals

Quercetin (HIMEDIA labs – Mumbai, India) was administered by gavages in propylene glycol as a vehicle. Gavages administrations were made 24 h and 1 h prior to the Cyclophosphamide intraperitoneal injection. Control animals were given propylene glycol vehicle only. Quercetin was administered at the doses level of 250 mg/kg. Cyclophosphamides (HIMEDIA labs – Mumbai, India) were dissolved in saline immediately before use. All other chemicals such as, DNA, RNA, Diphenyl Amine, Sodium Citrate & Orcinol

2.3. Experimental Protocol

Male mice were acclimatized for 2 days and divided into 4 groups consisting of 5 mice each, set up as follows: Group 1: mice were served as a control group and treated daily with the vehicle only for 15 days; Group 2: mice were injected with a single dose of 40 mg/kg Cyclophosphamide alone Group 3: mice were treated with quercetin in a dose of 250 mg/kg, once a day, for 15 days

followed by CYP induction on the day 15th. Group 4 mice were treated with quercetin in a dose of 250 mg/kg, respectively, once a day, for 15 days.

DNA Extraction.

1. Add 25-100 mg of Liver sample to 1.0 ml tube in a centrifuge tube. Vortex for 10 s at Full speed.

Buffer 1: Buffer Lysis (BL)

Note: Only use sterile tubes for Liver cells that do not adhere strongly to surface.

2. Centrifuge for 10 min at 5000 rpm

3. Carefully remove the supernatant, leaving ~ of Tissue/pellet and add Buffer 1, 600 µl.

4. Homogenize with Micro Pestle and transfer the micro centrifuge tube to 60 Degree Celsius Water Bath. Now Centrifuge, Keep Supernatant.

5. Add 0.5 ml Buffer 2 (Binding Buffer) to the tube and vortex for 10 s at full speed to collect Sample

6. Centrifuge for 2 min at full speed in a micro centrifuge.

7. Carefully remove the supernatant, and pass through Column, Elution Based

8. Rinse the column with 250 µl 75% Ethanol and Centrifuge twice or Thrice

9. Now the dry spin is been done for 13000 RPM for 15 seconds.

10. Add 100 µl Buffer Elution or TE Buffer or Nuclease free Water and collect sample and mix by vortexing.

RNA Extraction

Magnetic Bead RNA Extraction

1. Add 101µl lysis/binding solution to specimen well.

2. Add 50 mg specimen to the lysis/binding solution. Shake for 30 sec

3. Add 20µl resuspended magnetic beads to each well shake for 4 min.

4. Capture RNA binding beads on magnetic stand for 2 min.

5. Discard supernatant. Remove plate from magnetic stand.

6. Add 100µl wash solution I Shake for 30 sec.

7. Pellet the beads for 1 min. and remove wash supernatant

8. Add 100µl wash solution II

9. Shake for 30 sec. Repeat wash II Procedure

10. Following the 2nd wash II step dry the beads by shaking vigorously for 2 min.

11. All residual ETOH must be removed in dry step

12. Add 50µl elution buffer and shake for 4 min.

13. Collect the beads on the magnetic stand and transfer RNA to tube

2.4. Parameters Tested

A. The DNA was estimated by the Diphenyl amine

- In a series of clean and dry test tubes labeled as S1 to S5 0.5 to 2.5ml of standard DNA solution was pipette out with a concentration range of 250-1250 micro gm of DNA.
- The given unknown solution was made up to 100ml with saline sodium citrate solution.
- 1ml and 2 ml of the unknown solution was taken in tubes labeled as U1 and U2. 3ml of saline sodium citrate buffer alone served as blank.
- 5ml of diphenylamine reagent was added to all the tubes.

- The test tubes were incubated in boiling water bath for 10 minutes.
- The intensity of the blue color developed was read at 595 nm using red filter.
- A standard graph was constructed with absorbance at 595nm on Y-axis and concentration of DNA on X-axis. From the standard graph, the amount of DNA present in the unknown solution was calculated.

B. The RNA was estimated by the Orcinol Method:

- Pipette out standard RNA solution in arrange of 0–2 mL into a series of test tube and make up the volume of each tube 2 mL with distilled water.
- Add 3 mL of orcinol reagent to each tube for standard solution.
- For test solution: Take 2 mL of nucleic acid sodium (isolated from tissue source.) Add 3 mL of orcinol reagent to each tube, and heat the tube on boiling water bath for 20 minutes.
- Cool and take the optical density at 665 nm against the orcinol blank.

C. The protein was estimated by the Lowry method

- Different dilutions of BSA solutions are prepared by mixing stock BSA solution (1 mg/ml) and water in the test tube as given in the table. The final volume in each of the test tubes is 5 ml. The BSA range is 0.05 to 1 mg/ml.
- From these different dilutions, pipette out 0.2 ml protein solution to different test tubes and add 2 ml of alkaline copper sulphate reagents (analytical reagent). Mix the solutions well.
- This solution is incubated at room temperature for 10 min. Then add 0.2 ml of reagent Folin Ciocalteau solution (reagent solutions) to each tube and incubate for 30 min. Zero the colorimeter with blank and take the optical density (measure the absorbance) at 660nm.

LPO, GSH, MDA & SOD activities were shown in the table -2

The fragmentation of the DNA was observed by the DNA Gel Electrophoresis:

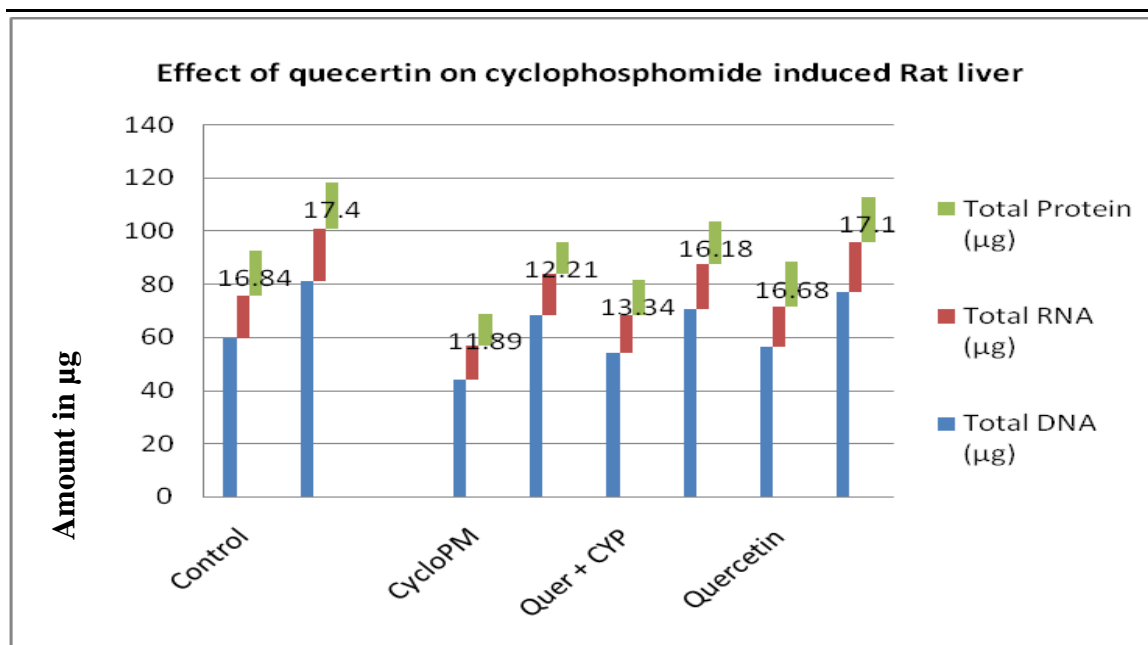
Normal gel electrophoresis principle has been followed.

D. Result and Discussion

Table 1. Effect of quercetin on cyclophosphomide induced Rat liver after fifteen days

| Sl. No | Treatment Dose | Total DNA (µg) | Total DNA (µg) | Total RNA (µg) | Total RNA (µg) | Total Protein (µg) | Total Protein (µg) |
|--------|---|----------------|----------------|----------------|----------------|--------------------|--------------------|
| 1 | Control (propylene glycol vehicle only) | 70.6 ± 10.86 | 59.74 81.46 | 17.9± 1.80 | 16.1 19.7 | 17.12 ± 0.28 | 16.84 17.4 |
| 2 | Cyclophosphomide (40 mg/kg/day) | 56.1 ± 12.25* | 43.85 68.35 | 14.4± 1.20^ | 13.2 15.6 | 12.05 ± 0.16# | 11.89 12.21 |
| 3 | Quercetin + CYP | 62.4 ± 8.22 | 54.18 70.62 | 15.6± 1.46 | 14.14 17.06 | 14.76 ± 1.42 | 13.34 16.18 |
| 4 | Quercetin (250mg/kg/day) | 66.8 ± 10.28* | 56.52 77.08 | 17.0± 1.72^ | 15.28 18.72 | 16.89 ± 0.21# | 16.68 17.1 |

Effect of Quercetin on Cyclophosphamide Induced Biochemical Profiles in Rat Liver



Note: Values are expressed as mean ± standard error with superscripts indicate a significant difference ($P < 0.05$). *#^

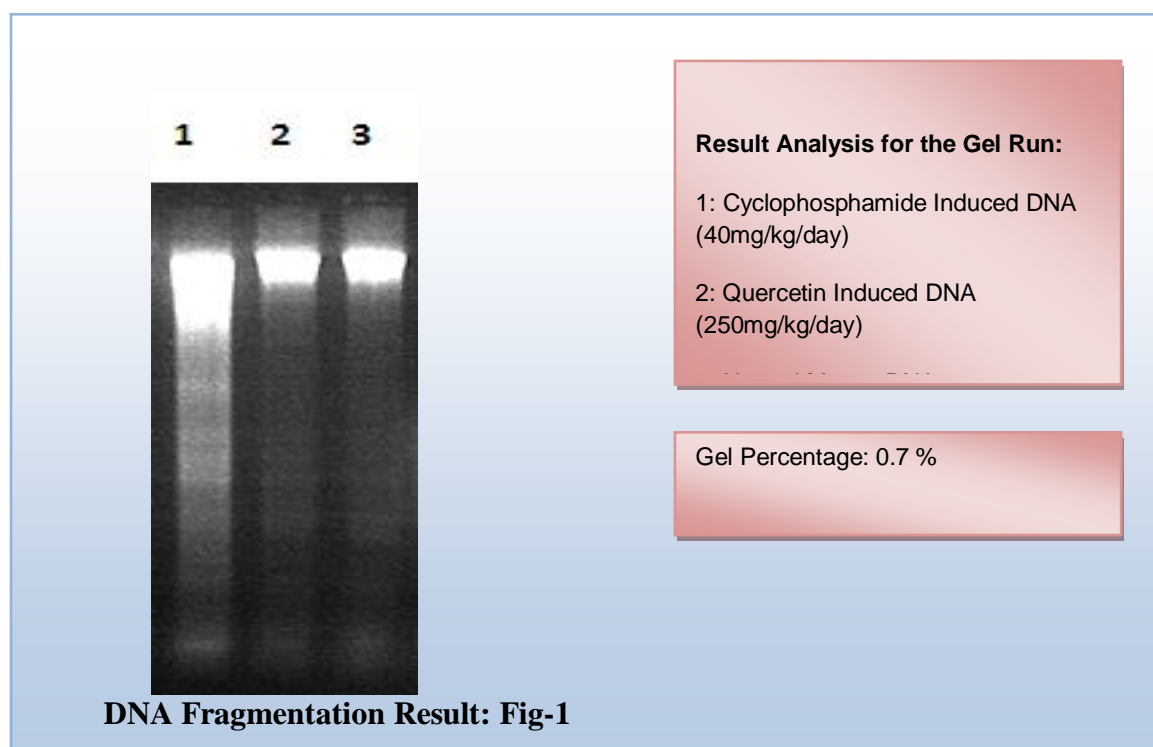


Table 2. The Effect of Quercetin (QE) on Cyclophosphamide (CP)-Induced Alterations in Lipid Peroxidase (LPO): expressed as MDA (μM /g protein), SOD (U/g protein) activities in rat liver ($n = 6$ per group). Values are expressed as mean ± standard error. In each row, values with different superscripts indicate a significant difference ($P < 0.05$).

| Groups | LPO | GSH | MDA | SOD |
|---------------------------------|-------------|------------|-------------|--------------|
| Control | 49.2 ± 3.4 | 1.4 ± 2.0 | 18.26±0.42 | 124.12±1.76 |
| Cyclophosphamide (40 mg/kg/day) | 82.8 ± 8.9* | 0.8 ± 0.2* | 16.08±0.56* | 131.52±2.40* |
| Quercetin + CP | 54.0 ± 6.0* | 1.3 ± 0.6* | 28.40±0.42* | 102.26±1.72* |
| Quercetin (250mg/kg/day) | 45.6 ± 4.9 | 1.5 ± 0.4 | 30.16±0.64 | 90.24±1.84 |

Normal control mouse was showed standard values. Where as after treatment for 15 days these was a decrease in total RNA content and total protein content for stressed or induced mouse were observed. There was a very little change in the total DNA content of liver cells which was found to be contributed due to fragmentation of DNA expressed in gel electrophoresis. In case of quercetin dosed mice there was elevation in the total amount of total RNA and total protein up to a significant level. The total DNA content was also showed to increase up to almost normal level. Gel electrophoresis also approves the estimation results as almost normal band was observed (Fig-1).

3. RESULT AND CONCLUSION

An increased production of free radicals via cyclophosphamide induced and non-enzymatic protein glycation which may lead to disruption of cellular functions and oxidative damage to membranes. Free radicals affect the cells components such as lipid, protein, DNA and carbohydrates, of which lipids are the most sensitive part. Hence, we determined the malondialdehyde (MDA) levels as the stable end product of lipid peroxidation. The levels of reactive oxygen species are controlled by antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and non-enzymatic scavengers, such as reduced glutathione (GSH). Earlier reports indicate increased or decreased lipid peroxidation, SOD, GSH-Px and CAT activities in various tissues, such as liver, kidney, blood vessels, heart, lymphoid organs, lungs and uterus. Thus, the tissue antioxidant status seems to have an important role in the etiology of metabolism. Mitochondrial oxidant stress and radicals formation has been implicated in the pathophysiology of cyclophosphamide induced (CYP-induced) liver injury. Therefore, we tested the hypothesis that lipid peroxidation (LPO) might be involved in the injury mechanism as its been examined that the LPO value in the control was found to be 49.2 ± 3.4 , but Peroxidation increased is by the Cyclophosphamide and its value is found to be $82.8 \pm 8.9^*$. However Quercetin alteration significantly reduced the Lipid peroxidation and LPO reaches to 45.6 ± 4.9 i.e. the control value. Both reduced GSH and MDA after CYP addition affects hepatotoxicity. Thus, despite the previously shown mitochondrial oxidant stress and radicals formation, LPO does not appear to be a critical event in CYP induced hepatotoxicity as GSH and MDA. To verify a potential effect of the Quercetin diet induced liver injury, animals were pretreated with a Quercetin. We observed, massive control in the LPO, GSH, MDA & SOD in liver cell injury in the animals were controlled, as indicated in **table 2**. Based on the study performed on the Rat, it has been concluded that the Drugs Cyclophosphamide is a stress inducing molecule which is directly targeting on the Nucleic acid contents that is the DNA & RNA. However the Quercetin has been noticed for the anti stress remedy that is it is providing the free radical activity.

ACKNOWLEDGEMENT

The authors are immense grateful to the Dr.Hemachandra Sagar (Chairman), Dr.Prema Chandra Sagar (Vice Chairman) and Dr.Krishne Gowda (Director of the Biological Science) of Dayananda Sagar Institutions, Bangalore, INDIA

REFERENCES

- [1] Alexander SP: Flavonoids as antagonists at A1 adenosine receptors. *Phytother Res* 2006, 20:1009-1012.
- [2] Azuma K, Ippoushi K, Terao J: Evaluation of tolerable levels of dietary quercetin for exerting its antioxidative effect in high cholesterol-fed rats. *Food Chem Toxicol* 2010, 48:1117-1122.
- [3] Bowman C, Peters DK, Lockwood CM. Anti-glomerular basement membrane autoantibodies in the Brown Norway rat: detection by a solid-phase radioimmunoassay. *J Immunol Methods*. 1983 Jul 29;61(3):325–333.
- [4] Chevront SN, Ely BR, Kenefick RW, Michniak-Kohn BB, Rood JC, Sawka MN: No effect of nutritional adenosine receptor antagonists on exercise performance in the heat. *Am J Physiol Regul Integr Comp Physiol* 2009, 296:R394-R401.
- [5] Cupps TR, Edgar LC, Fauci AS. Suppression of human B lymphocyte function by cyclophosphamide. *J Immunol*. 1982 Jun;128(6):2453–2457.

- [6] Davis JM, Murphy EA, Carmichael MD, Davis B: Quercetin increases brain and muscle mitochondrial biogenesis and exercise tolerance. *Am J Physiol Regul Integr Comp Physiol* 2009, 296:R1071-R1077.
- [7] Davis JM, Murphy EA, McClellan JL, Carmichael MD, Gangemi JD: Quercetin reduces susceptibility to influenza infection following stressful exercise. *Am J Physiol Regul Integr Comp Physiol* 2008, 295:R505-R509.
- [8] De Boer VC, Dihal AA, van der Woude H, Arts IC, Wolfram S, Alink GM, Rietjens IM, Keijer J, Hollman PC: Tissue distribution of quercetin in rats and pigs. *J Nutr* 2005, 135:1718-1725.
- [9] Ferré S: An update on the mechanisms of the psychostimulant effects of caffeine. *J Neurochem* 2008, 105:1067-1079.
- [10] Hardwood M, Danielewska-Nikiel B, Borzelleca JF, Flamm GW, Lines TC: A critical review of the data related to the safety of quercetin and lack of evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties. *Food Chem Toxicol* 2007, 45:2179-2205.
- [11] Lagouge M, Argmann C, Gerhart-Hines Z, Meziane H, Ierini C, Daussin F, Mesadeq N, Milne J, Lambert P, Elliott P, Geny B, Laakso M, Puigserver P, Auwerx J: Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1 α . *Cell* 2006, 127:1109-1122.
- [12] Manach C, Scalbert A, Morand C, Rémesy C, Jimenez L: Polyphenols: food sources and bioavailability. *Am J Clin Nutr* 2004, 79:727-747.
- [13] Middleton E, Kandaswami C, Theoharides TC: The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Rev* 2000, 52:673-751.
- [14] Nieman DC, Henson DA, Davis JM, Angela Murphy E, Jenkins DP, Gross SJ, Carmichael MD, Quindry JC, Dumke CL, Utter AC, McAnulty SR, McAnulty LS, Tripplett NT, Mayer EP: Quercetin's influence on exercise-induced changes in plasma cytokines and muscle and leukocyte cytokine mRNA. *J Appl Physiol* 2007, 103:1728-1735.
- [15] Nishimura t., yoshida y., watanabe f. Et al. Blood level of mitochondrial aspartate aminotransferase as an indicator of the extent of ischemic necrosis of the rat liver. *Hepatology*, 1986, 6: 701-7.
- [16] Sener g., tosun o., sehirlir a. O. Et al. Melatonin and N-acetylcysteine have beneficial effects during hepatic ischemia and reperfusion. *Life Sci*, 2003, 72 : 2707-18.
- [17] Su j. F., guo c. J., wei j. Y., yang j. J., jiang y. G., li y. F. Protection against hepatic ischemia-reperfusion injury in rats by Oral pretreatment with quercetin. *Biomed Environ Sci*, 2003, 16 :1-8.
- [18] Watson A.R., Rance C.P. and Bain J. (1985). Long term effect of cyclophosphamide on testicular function. *Br. Med. J. Clin. Res.* 291, 60.