UGC MAJOR RESEARCH PROJECT

FINAL YEAR REPORT

(UGC letter No. F.No. 37-46/2009 dated 12.01.2010)

on

CHRONOPHARMACOLOGICAL AND CHRONOTHERPEUTIC EFFECT OF FISETIN IN HYPERAMMONEMIC RATS

STATEMENT OF EXPENDITURE

&

UTILISATION CERTIFICATE

2010-2012

(01.02.2010 to 31.01.2013)

Submitted by

Dr. P. SUBRAMANIAN
Principal Investigator
UGC Major Research Project
Professor
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Annamalainagar – 608 002
Tamil Nadu.

ज्ञान-विज्ञान विमुक्तये

Annexure – IX

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI – 110 002.

THE FINAL REPORT OF THE WORK DONE ON THE PROJECT

1. **Name and address of the principal investigator** : **Dr. P. SUBRAMANIAN**
Professor,
Dept. of Biochemistry & Biotechnology
Faculty of Science, Annamalai University,
Annamalai Nagar-608 002,
Tamil nadu – India.

2. **Name and address of the institution** : Dept. of Biochemistry & Biotechnology
Faculty of Science, Annamalai University,
Annamalai Nagar – 608 002,
Tamil nadu – India.

3. **UGC approval No. and date** : UGC No. F.No. 37-46/2009 (SR) dated 12.01.2010

4. **Date of implementation** : 01.02.2010

5. **Tenure of the project** : Three years (from 01.02.2010 to 31.01.2013)

6. **Total Grant Allocated** : Rs. 13,91,085.00/-

7. **Total Grant Received** : Rs. 12,85,306.00/-

8. **Final Expenditure** : Rs. 12,83,786.00/-

9. **Title of the Project** : "Chronopharmacological and Chronotherapeutic effect of fisetin in Hyperammonemic rats"

10. **Objectives of the Project** :

The main aim of this study was to elucidate the antihyperammonemic potential and chronotherapeutic effect of fisetin by performing following studies:

(i) Chronotherapeutic effect (00:00, 06:00, 12:00 and 18:00h) of fisetin on lipid peroxidation products and antioxidants.

(ii) lipid profile in chronic hyperammonemic rats.

(iii) The amino acids of brain in chronic hyperammonemic rats.

(iv)To determine the temporal patterns of urea cycle enzymes by western blotting.

(v) To evaluate the effect of fisetin on histopathological changes in the liver and brain to conform its protective role (hepatoprotective and neuroprotective) in ammonium chloride intoxicated hyperammonemic rats.

11. **Whether objectives were achieved :**

Yes, the experiments were carried out according to the objectives mentioned in the proposal, also added some additional activity recordings and molecular analysis of some inflammatory markers to achieve the objectives completely and effectively.

12. **Achievements from the project**

(i) **Workshop organized :** Nil

(ii) Collaboration :

Locomotor activity recording (Part of the project work) were performed with the help of Dr.M.Singaravel, Associate professor, Department of Zoology, Banarus Hindu University, Varanasi- India.

Report of the work done : **Separate sheets attached**

13. **Summary of the findings**

In the present study we evaluated the chronotherapeutic pattern of antihyperammonemic effects on redox status, lipid profile, amino acids and urea cycle enzymes of fisetin (50mg/kg b.w) administered to rats at 06:00, 12:00, 18:00 and 24:00 h against ammonium chloride (AC) (100mg/kg: i.p) induced hyperammonemic Wistar rats (180-200g). Ameliorative effect of fisetin on AC induced hyperammonemia at different time points (06:00, 12:00, 18:00 and 24:00 h) was evaluated by analyzing the circulatory levels of ammonia, urea, uric acid, creatinine, bilirubin, liver marker enzymes such as aspartate transaminase (AST),

alanine transaminase (ALT), alkaline phosphatase (ALP) and γ-glutamyl transferase (GGT), lipid peroxidation products and antioxidants such as thiobarbituric acid reactive substances (TBARS), lipid hydroperoxides (LP), conjugated diene (CD), nitric oxide (NO), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH), lipid profile such as total cholesterol, free fatty acids, triglycerides and phospholipids, amino acids such as glutamine and glutamate were analyzed in the tissues (liver and brain), the Western blot analysis of inflammatory markers such as inducible nitricoxide synthase (iNOS), nuclear factor kappa-B (NF-κB), urea cycle enzymes such as carbamoyl phosphate synthetase–I (CPS-I), ornithine transcarbamoylase (OTC), argininosuccinate synthase (ASS) and glutamine synthetase (GS). The locomotor activity changes recorded by activity wheel running monitor (AWM) and histopathological analysis were also performed with tissues (liver and brain).

The increased levels of ammonia, urea, uric acid,creatinone, bilirubin, liver marker enzymes (AST,ALT,ALP and GGT), lipid peroxidation products (TBARS, HP and CD) and decreased levels of antioxidants (SOD, CAT, GPx and GSH) and lipid profiles were observed in AC treated rats but the condition was upturned in treatment with fisetin. Fisetin reduced the fragmentation in the night time activity in hyperammonemic rats , this might be due to the neuromodulatory effect of fisetin on central nervous system. Fisetin administration at 24:00 h shows significant effects on those parameters than the other time points ($p<0.05$) DMRT. Thus, this may be due to pharmacokinetic property of the drug (fisetin) on temporal variations of levels and activity of urea cycle enzymes, lipid peroxidation and antioxidants, inflammatory markers.

14. Contribution to the society

Circadian (about 24h) rhythms are known to regulate hundreds of function of the body including blood pressure hormone production, digestive secretion, immune activity, DNA synthesis and cell division. Disruptions of these rhythms have profound influence on health. Therapeutic index of more than 300 drugs can be modified by varying the time of administration in a variety of diseases. The cellular functions of healthy tissues as well as the pharmacologic and toxicologic effects of drugs are characterized by circadian rhythms with predictable times of peak and trough. The chronotherapy concept offers further promise for improving current dysfunctions by minimizing undesirable side effects as well as by optimizing the efficacy of

drugs. The results of the proposal will add more knowledge on the function of biological clocks and cellular level during hyperammonemia, hepatic and brain encephalopathic condition. The substantial progresses have been made concerning knowledge of bioactive components of food ingredients, plants, phytochemicals and their links to health and diseases. Hence the antioxidant therapy is gaining significance in liver diseases, neurological defects, cornonary heart diseases, atherosclerosis and inflammation. Thus, the chronoparmocological studies on fisetin for further clinical trials would have important implications in trailoring novel ways of chronotherapy of hyperammonemia/liver dysfunction.

15. Whether any Ph.d. Enrolled/produced out of the project

- One Ph.D enrolled

16. No. of publications out of the project

(i) One research article bublished in the journal of biological rhythm research entitled "Chronotherapeutic effect of fisetin on Ammonium chloride induced hyperammonemic rats". *Biological rhythm research*, 2012; 1-12. Doi:10.1080/0929.2012.730890. **(Re-print attached)**

(ii) One research articles were communicated

PRINCIPAL INVESTIGATOR

Date: 31.03.2013

Dr. P. SUBRAMANIAN
PRINCIPAL INVESTIGATOR
UGC PROJECT
DEPARTMENT OF BIOCHEMISTRY
FACULTY OF SCIENCE
ANNAMALAI UNIVERSITY
ANNAMALAINAGAR 608 002

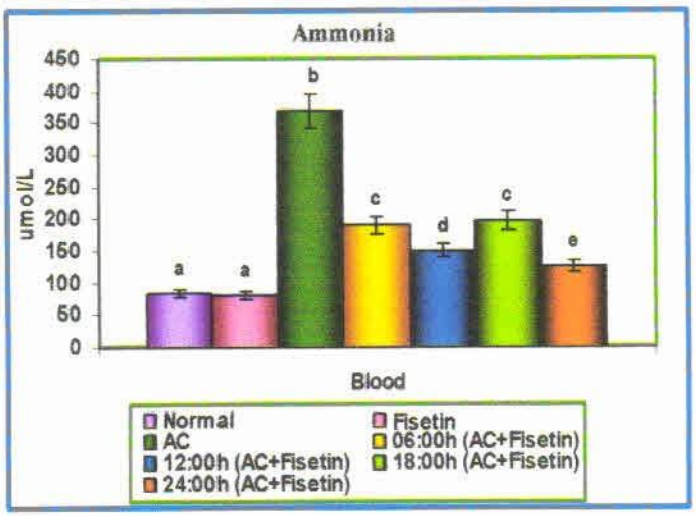
REGISTRAR

REGISTRAR

RESULTS

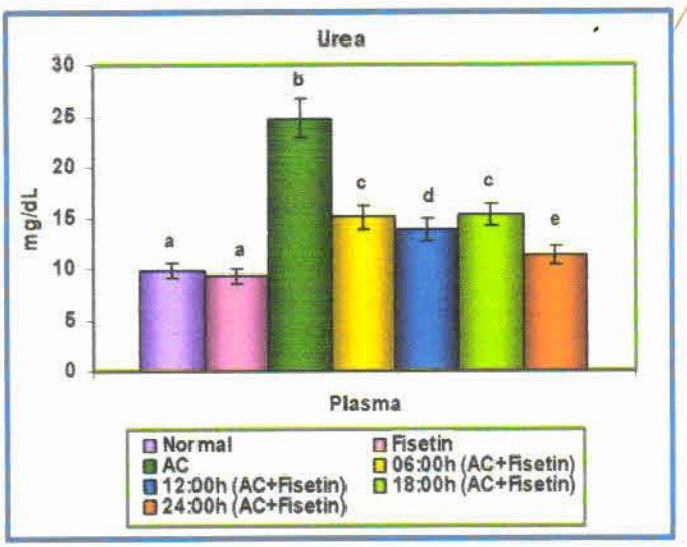
Liver and Kidney markers

Figure .1. Effect of Fisetin on changes in the blood ammonia of normal and experimental rats



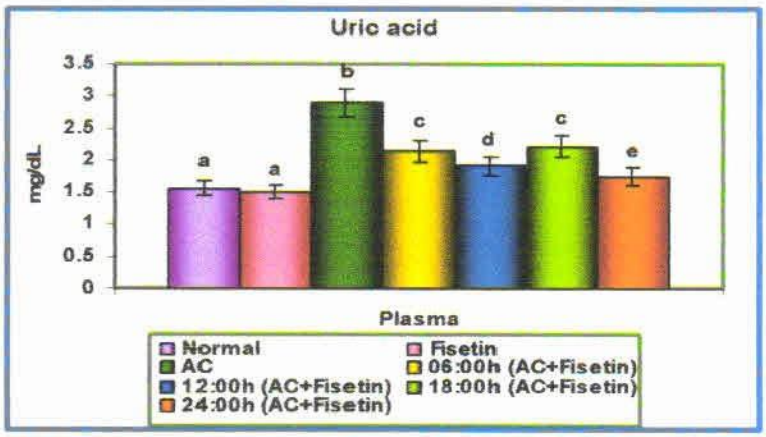
Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

Figure .2. Effect of Fisetin on changes in the plasma urea of normal and experimental rats



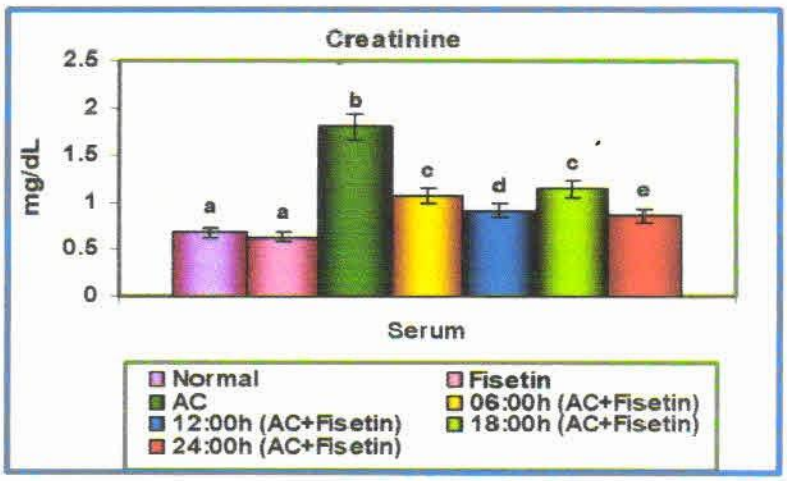
Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

Figure .3. Effect of Fisetin on changes in the plasma Uric acid of normal and experimental rats



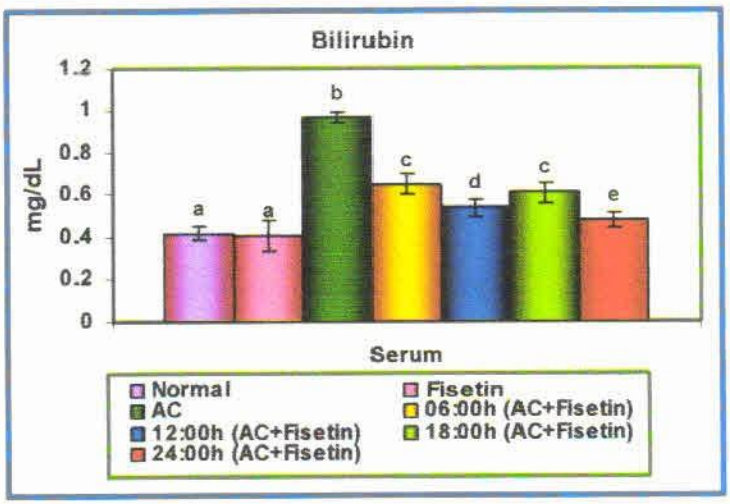
Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

Figure.4. Effect of Fisetin on changes in the Serum creatinine of normal and experimental rats



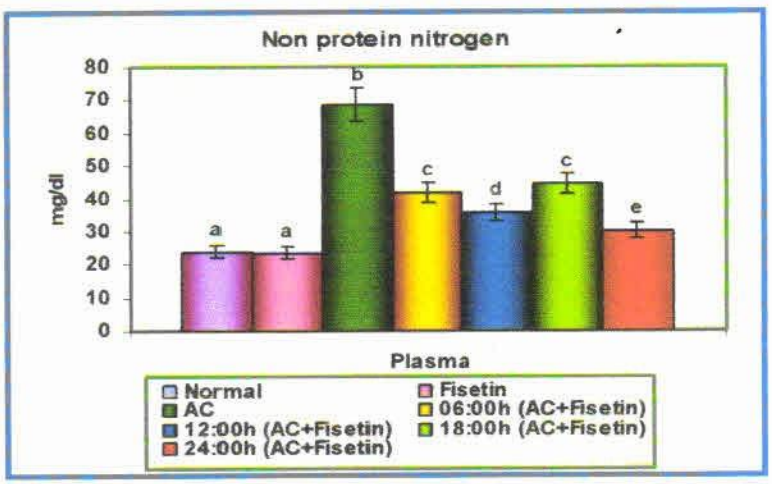
Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

Figure .5. Effect of Fisetin on changes in the Serum Bilirubin of normal and experimental rats



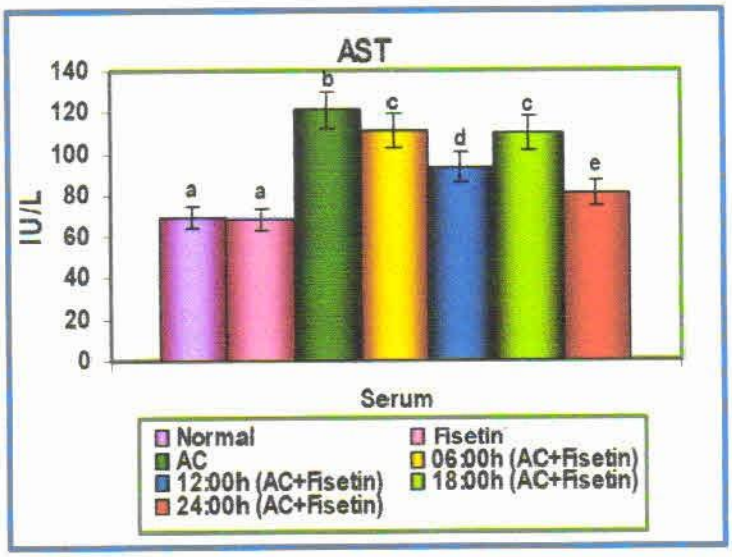
Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

Figure .6. Effect of Fisetin on changes in the Plasma non protein nitrogen of normal and experimental rats

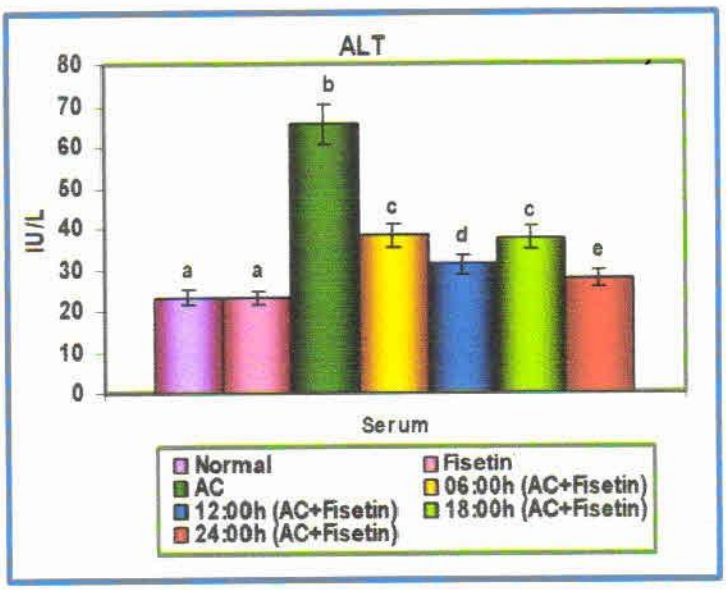


Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

Figure .7. Effect of Fisetin on changes in the activity of AST and ALT in normal and experimental rats

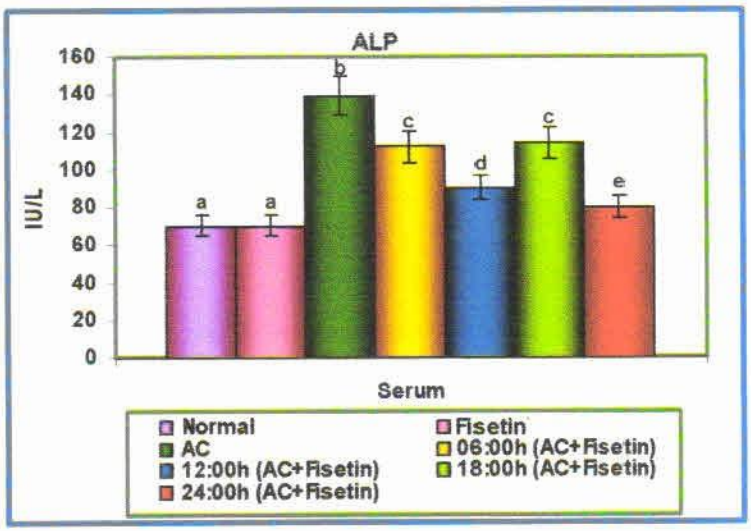


Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

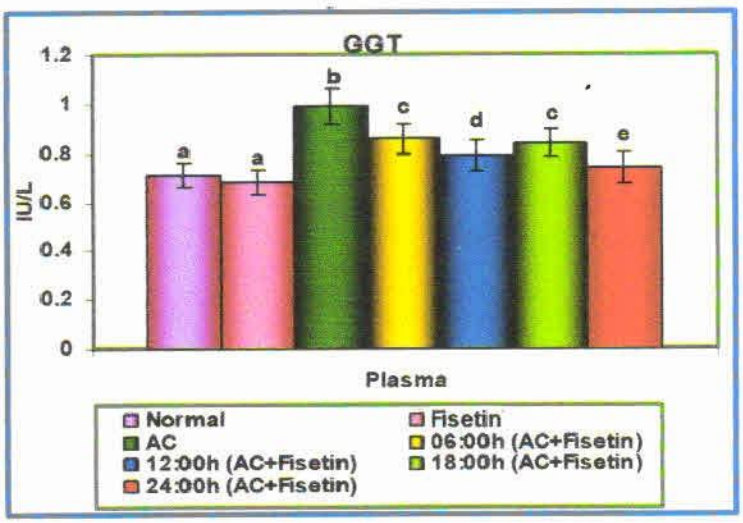


Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

Figure .8. Effect of Fisetin on changes in the activity of ALP and GGT in normal and experimental rats



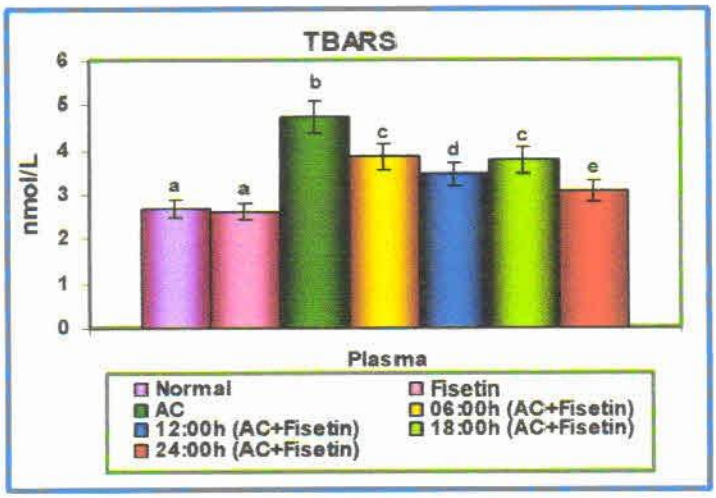
Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)



Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

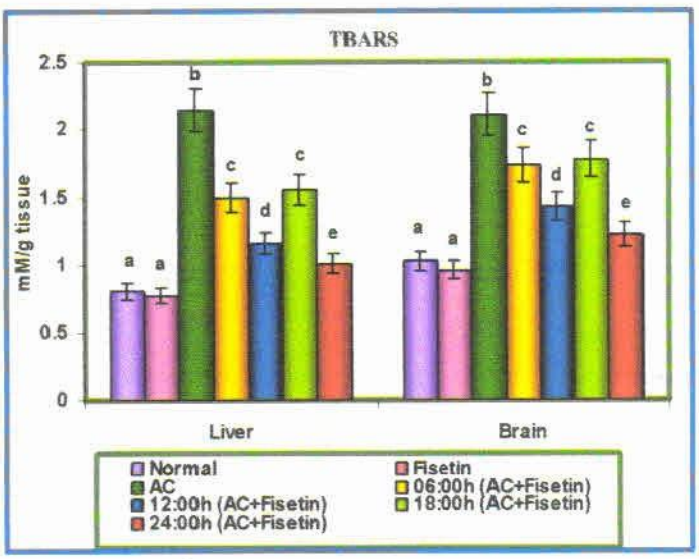
Lipid peroxidation and Antioxidants

Figure .9. Effect of Fisetin on changes in the plasma TBARS of normal and experimental rats



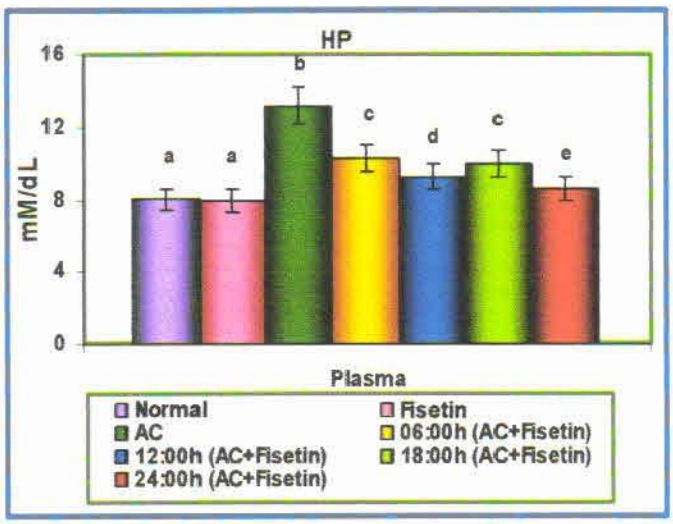
Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

Figure .10. Effect of Fisetin on changes in the liver and brain TBARS of normal and experimental rats

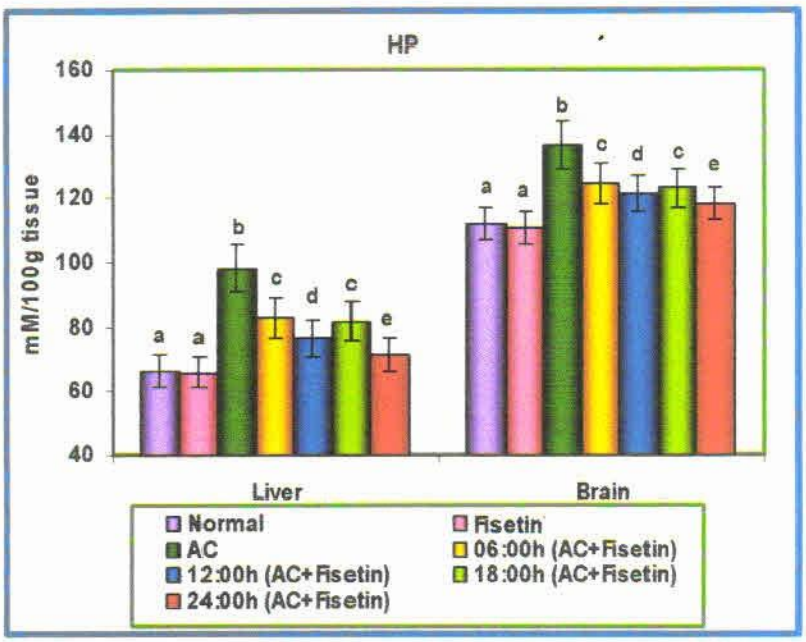


Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

Figure.11. Effect of Fisetin on changes in the levels of plasma and tissues (liver and brain) HP in normal and experimental rats

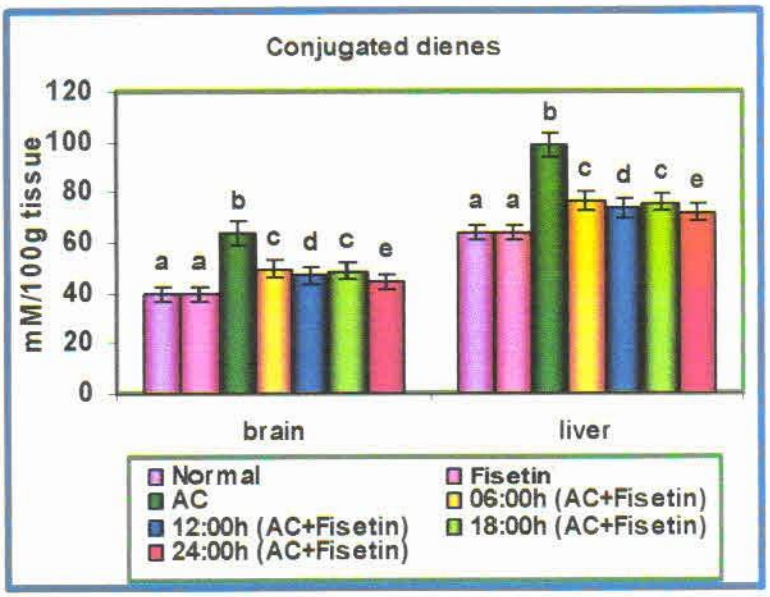


Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)



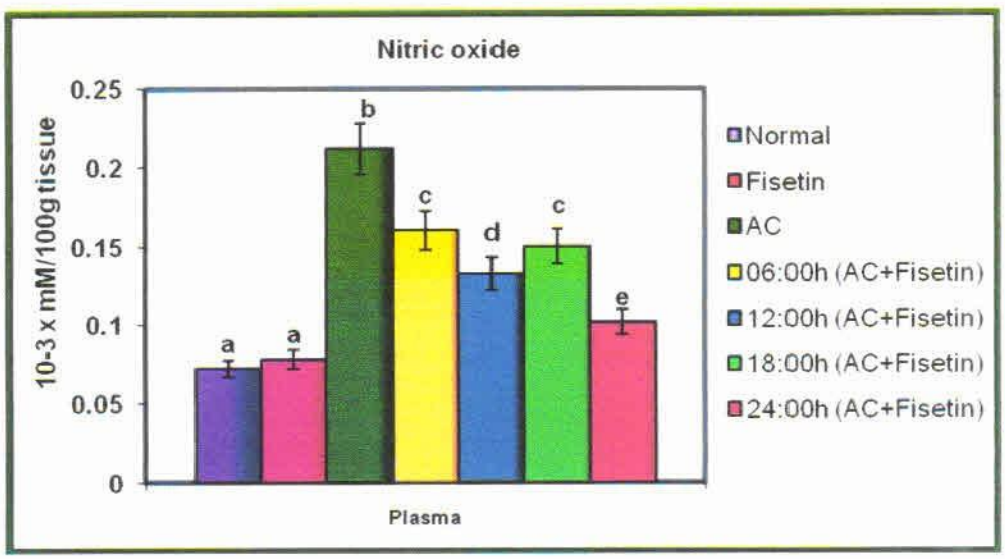
Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

Figure.12. Effect of Fisetin on changes in the conjugated dienes in tissues (Brain and Liver) of normal and experimental rats

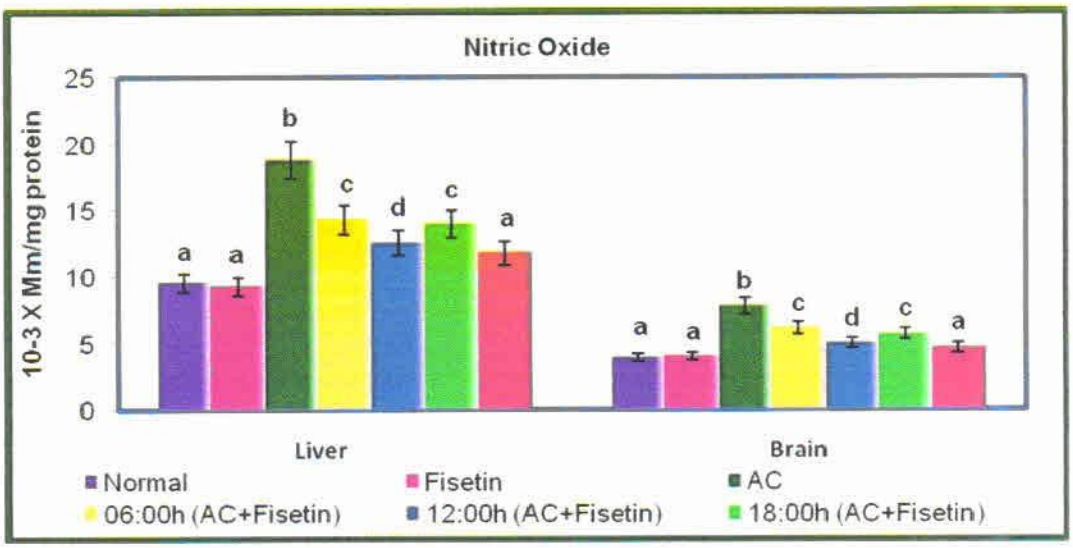


Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

Figure.13. Effect of Fisetin on changes in the Nitric oxide (NO) in plasma and tissues (liver and brain) of normal and experimental rats

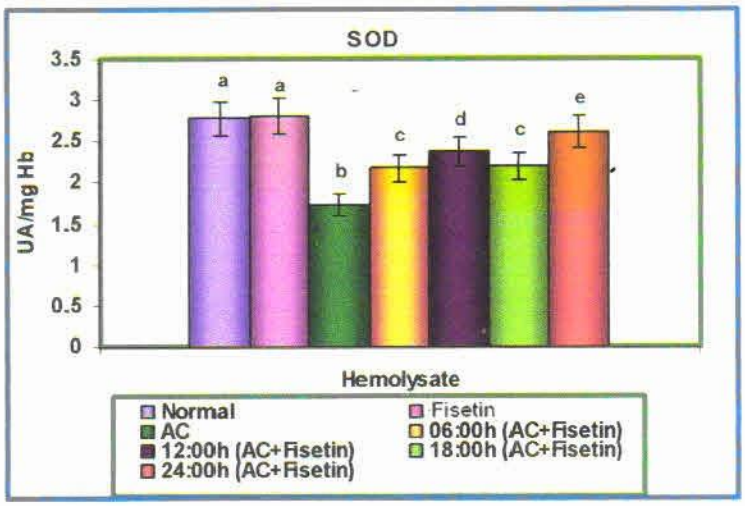


Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

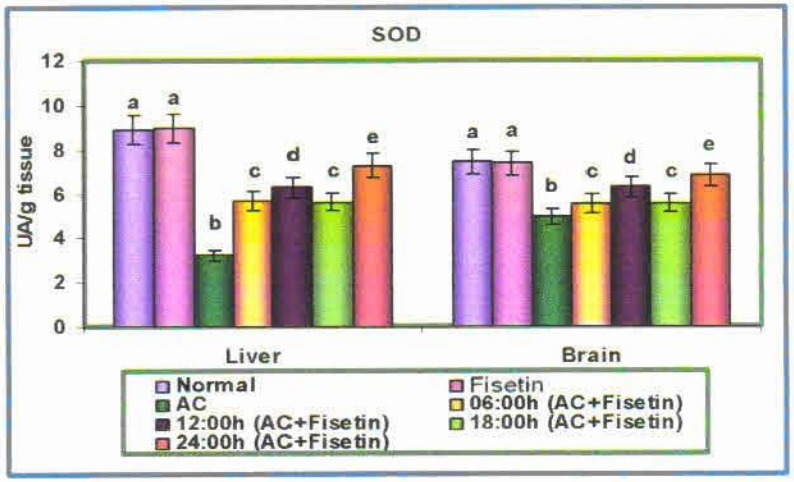


Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

Figure .14. Effect of Fisetin on changes in the activity of SOD in hemolysate ant tissues (Liver and Brain) of normal and experimental rats

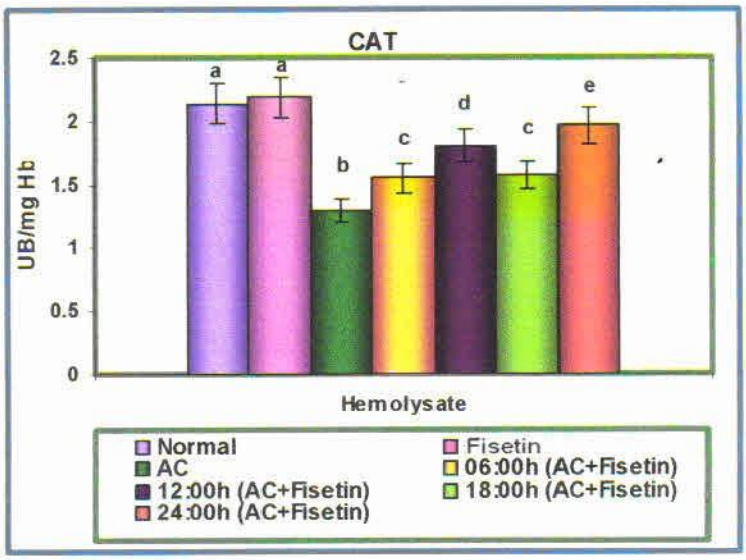


Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)
U^A - one unit of activity was taken as the enzyme reaction, which gave 50% inhibition of NBT reduction in 1 minute

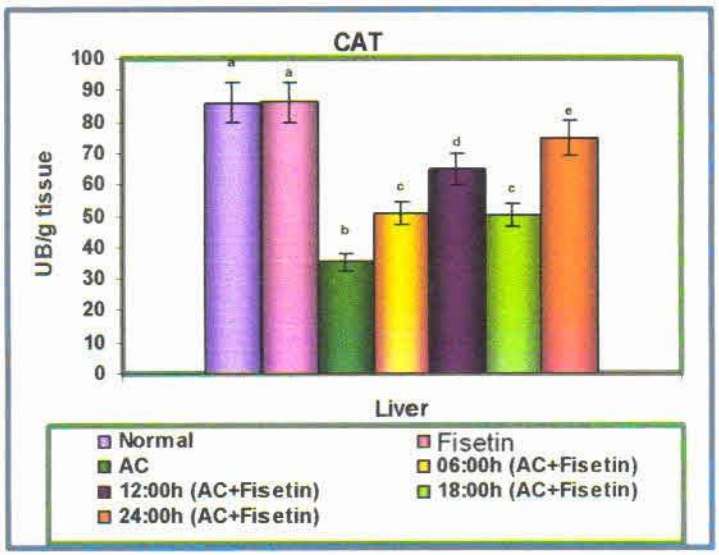


Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)
U^A - one unit of activity was taken as the enzyme reaction, which gave 50% inhibition of NBT reduction in 1 minute

Figure .15. Effect of Fisetin on changes in the activity of CAT in hemolysate ant tissues (Liver and Brain) of normal and experimental rats

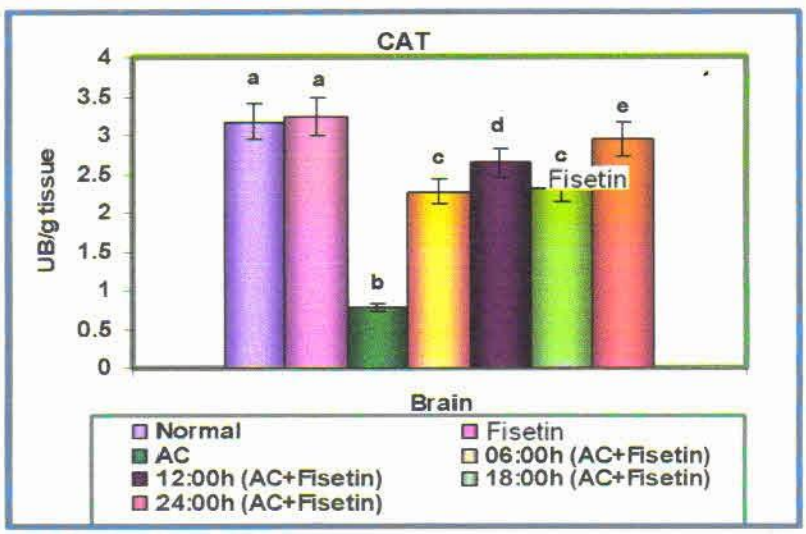


Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)
U^B - μ mole of hydrogen peroxide consumed/minute



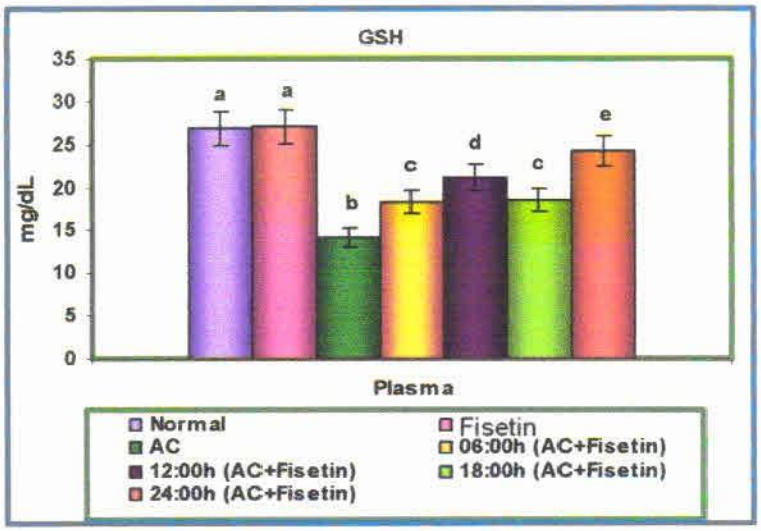
Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)
U^B - μ mole of hydrogen peroxide consumed/minute

Figure .16. Effect of Fisetin on changes in the activity of CAT in Brain of normal and experimental rats



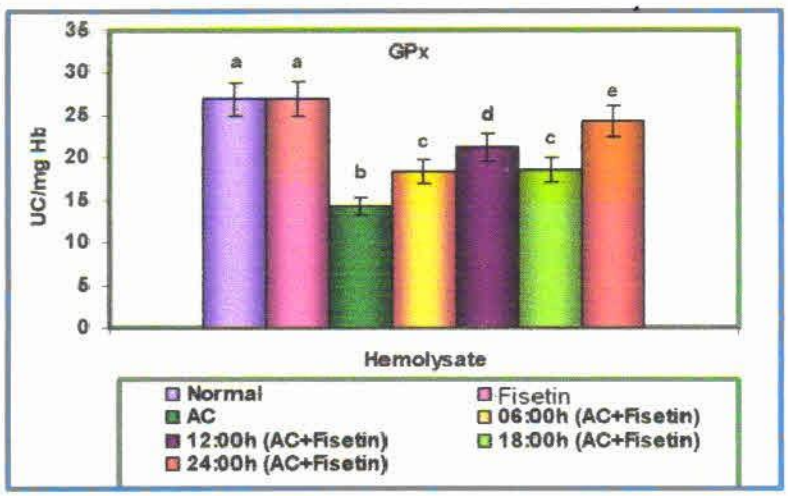
Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)
U^B - μ mole of hydrogen peroxide consumed/minute

Figure.17. Effect of Fisetin on changes in the activity of plasma GSH of normal and experimental rats

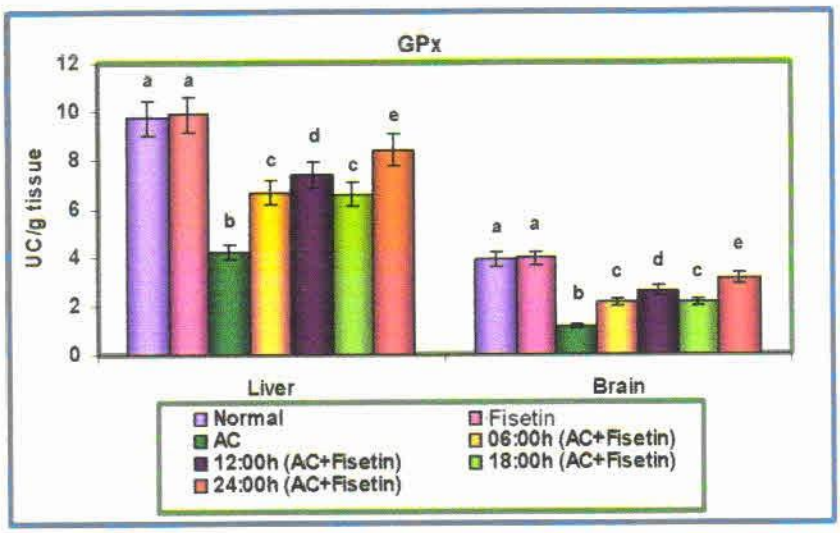


Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

Figure .17. Effect of Fisetin on changes in the activity of GPx in hemolysate ant tissues (Liver) of normal and experimental rats



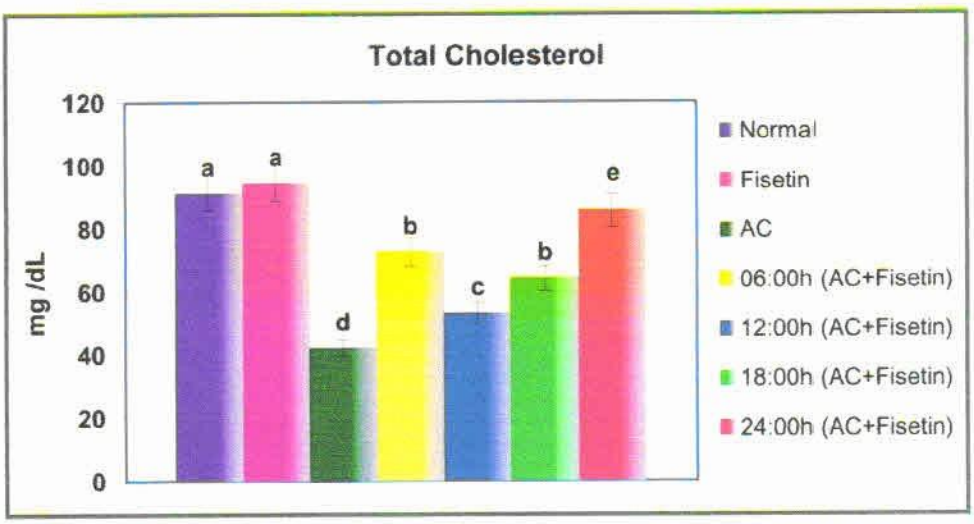
Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)
U^C - µg of glutathione consumed/minute



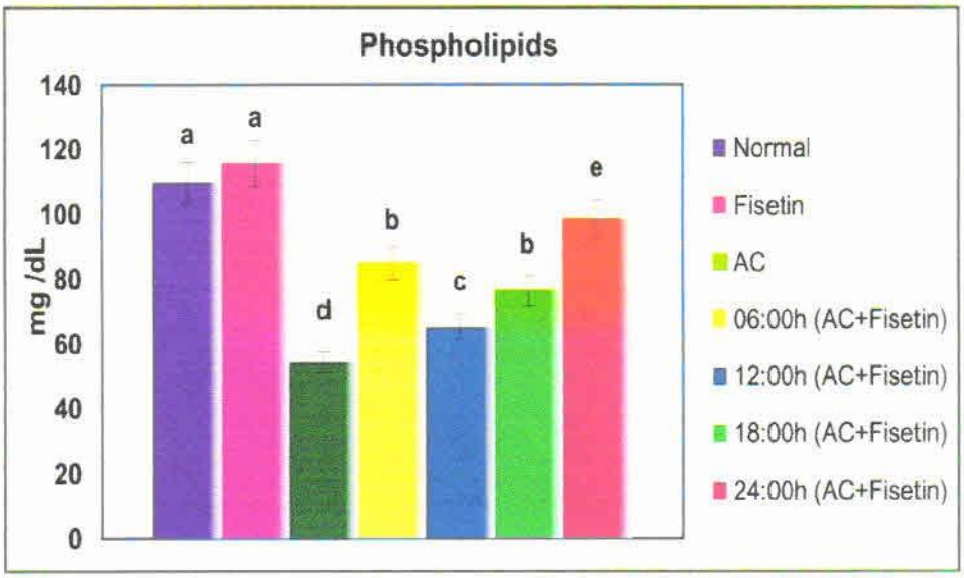
Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)
U^C - µg of glutathione consumed/minute

Lipid profile

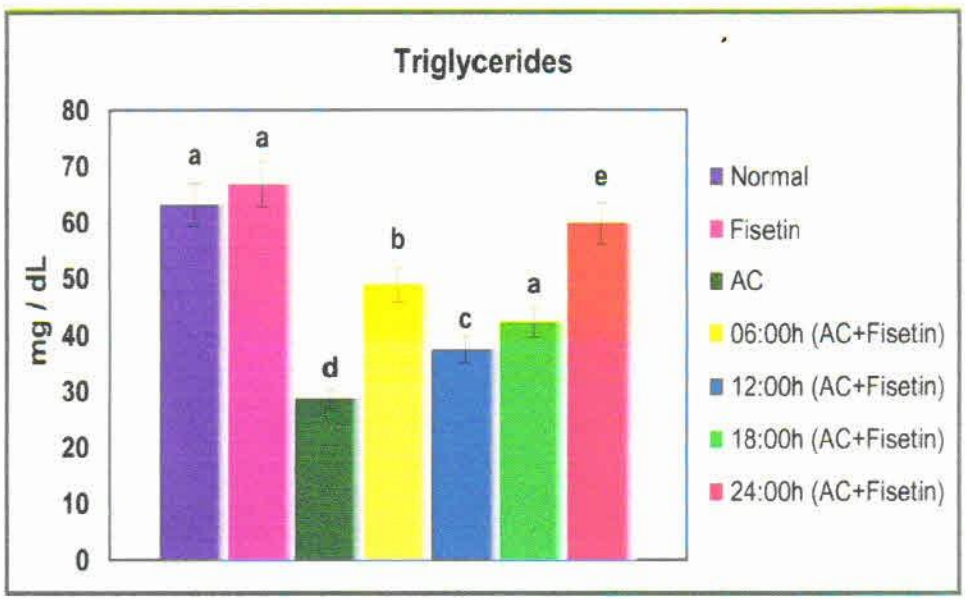
Figure .18. Effect of Fisetin on changes in the serum total cholesterol, phospholipids, free fatty acids and triglycerides of normal and experimental rats



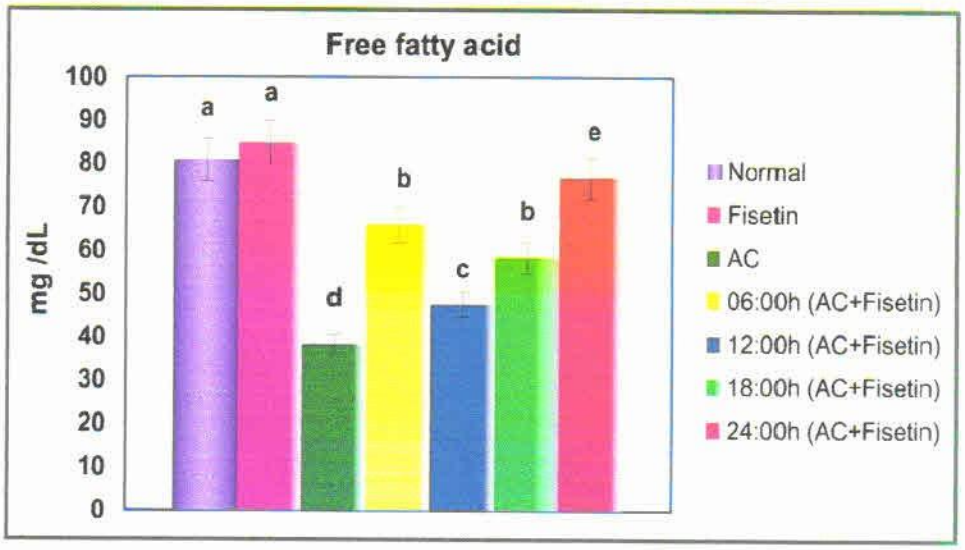
Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)



Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

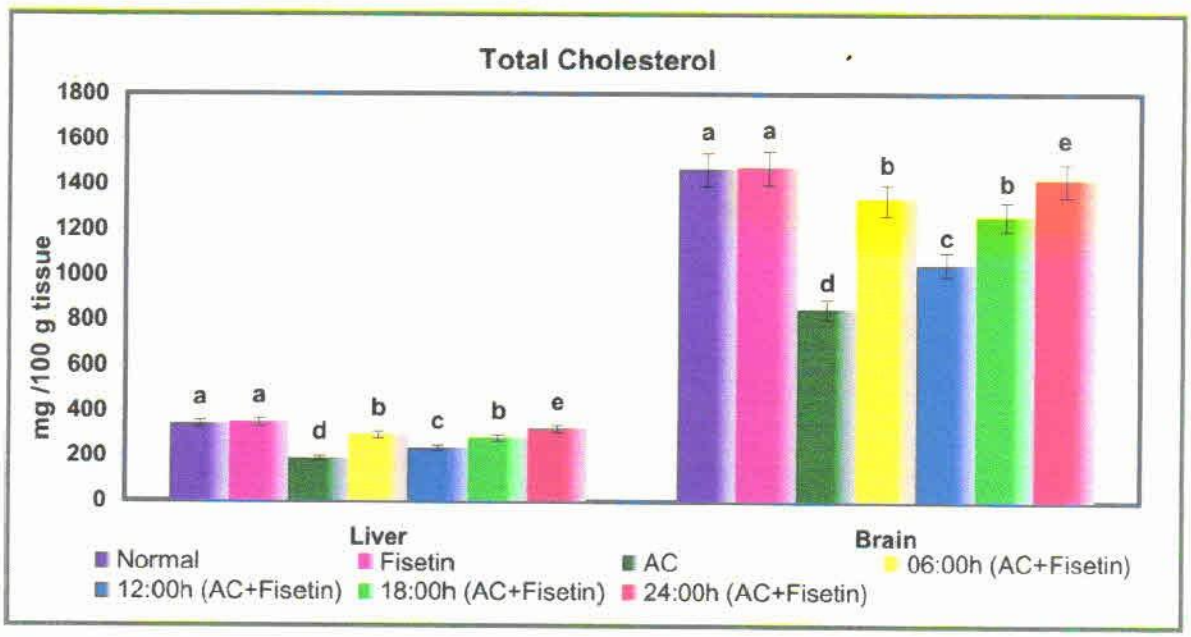


Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

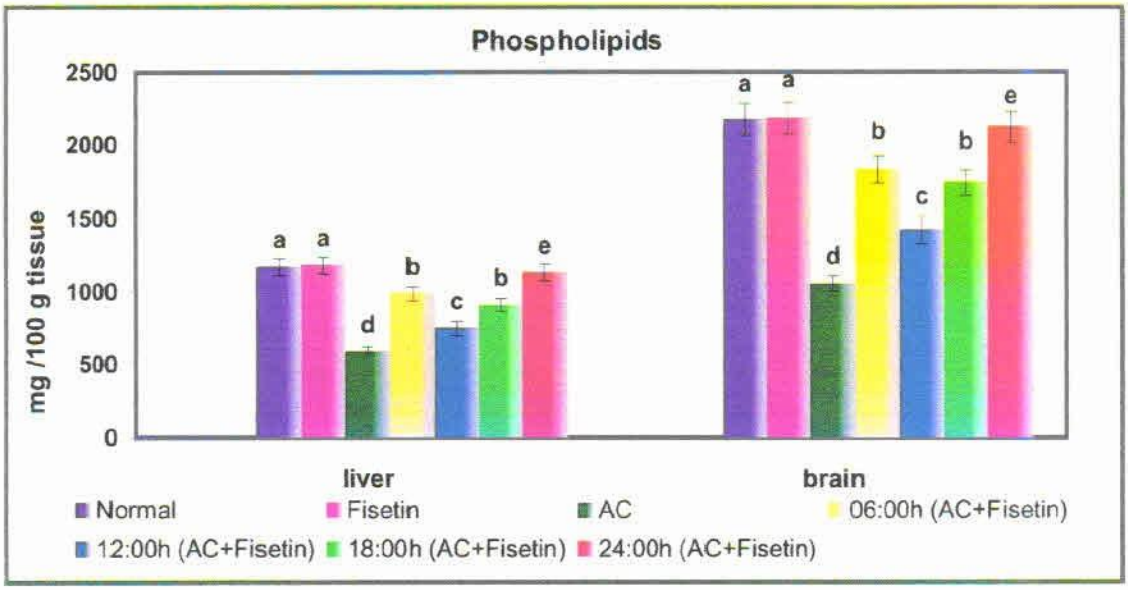


Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

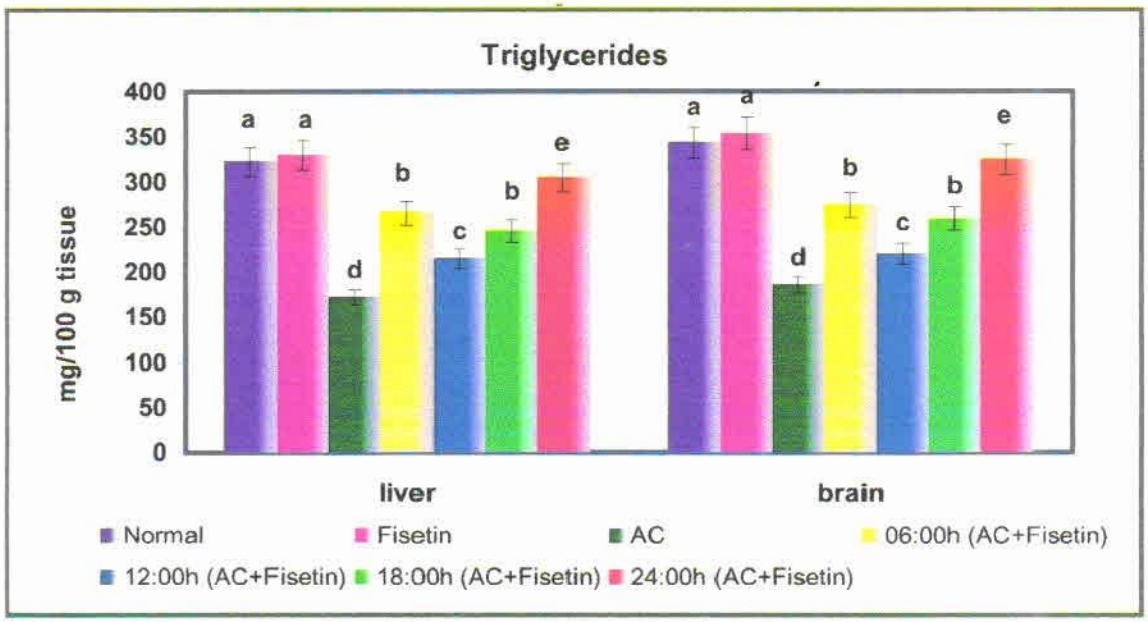
Figure .19. Effect of Fisetin on changes in the total cholesterol, phospholipids, free fatty acids and triglycerides in the tissues (liver and brain) of normal and experimental rats



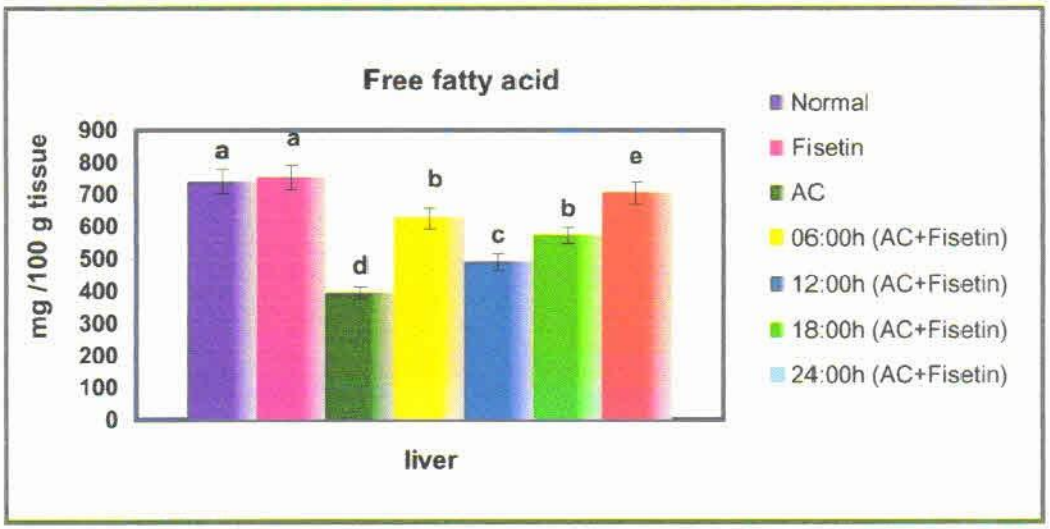
Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)



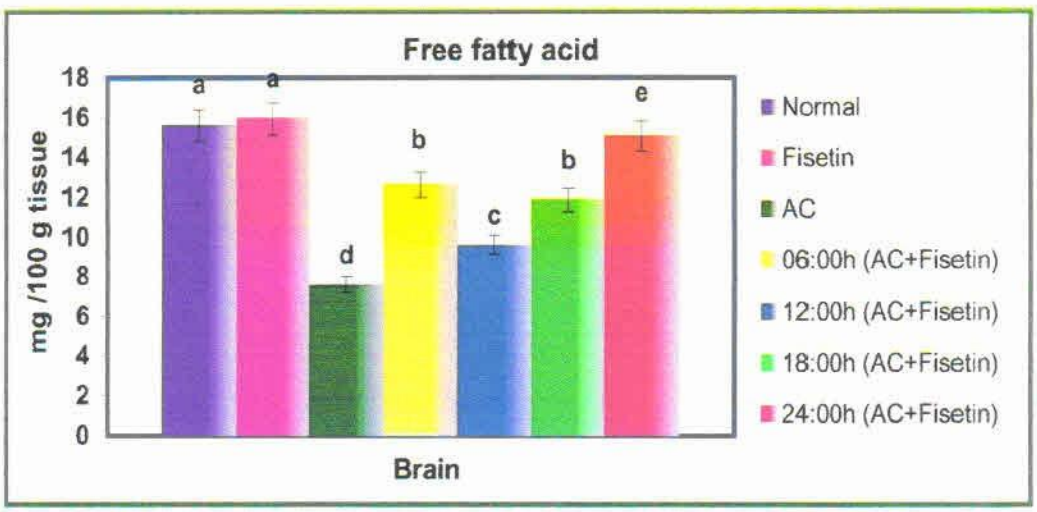
Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)



Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)



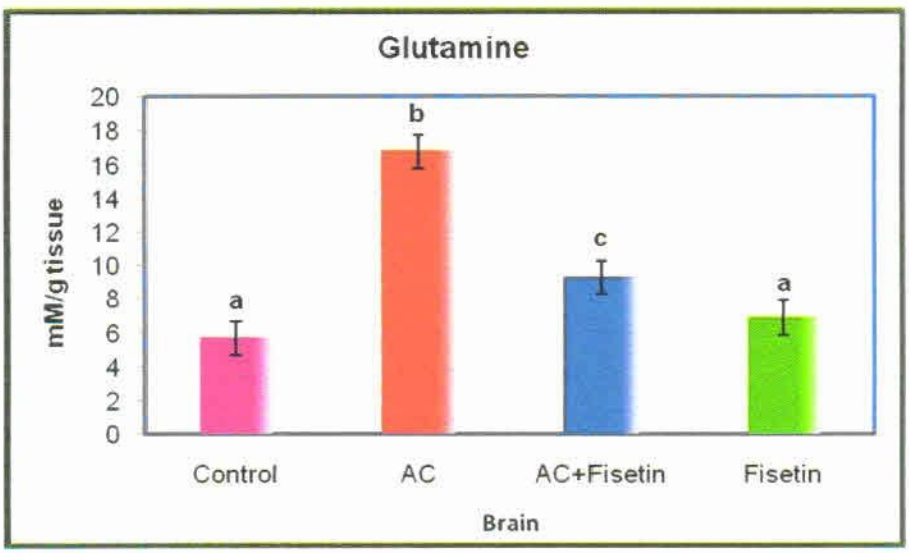
Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)



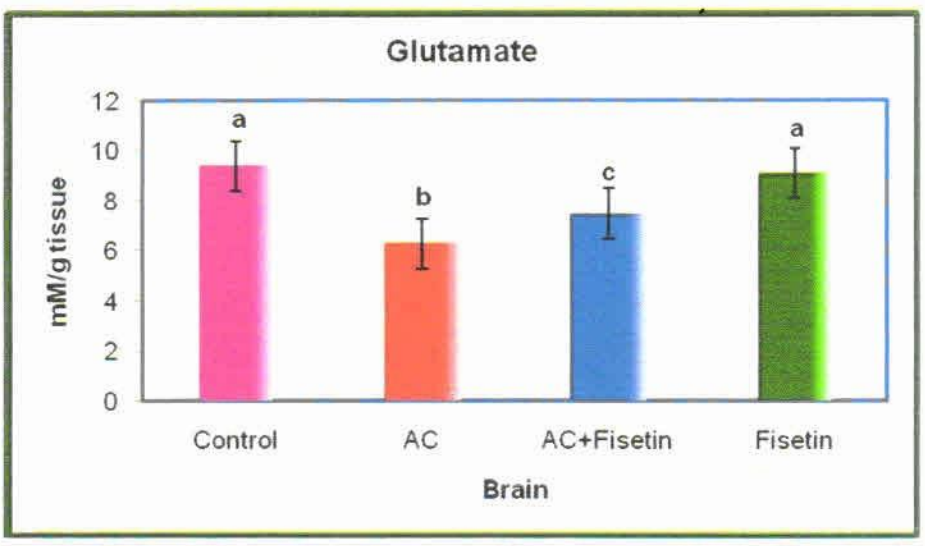
Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

Amino acids

Figure .20. Effect of Fisetin on changes in the glutamine and glutamate in the tissue (brain) of normal and experimental rats



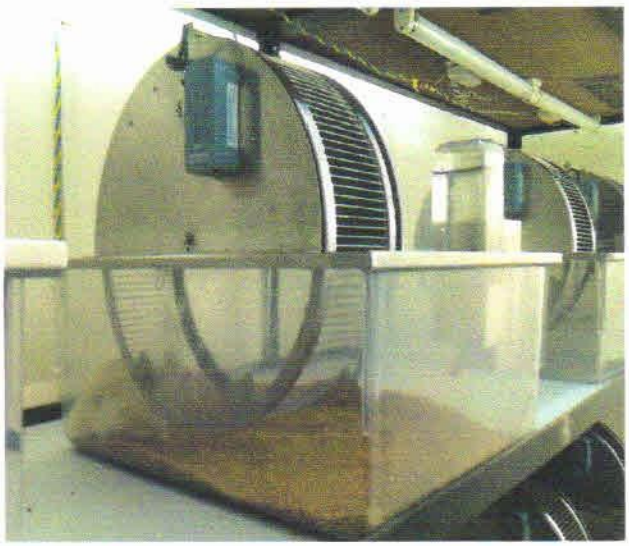
Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)



Values are given as mean ± S.D from eight rats in each group
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT)

LOCOMOTOR ACTIVITY

(Activity wheel running monitor (AWM))



Double Plotted Actogram

Figure 21.a: Control rats

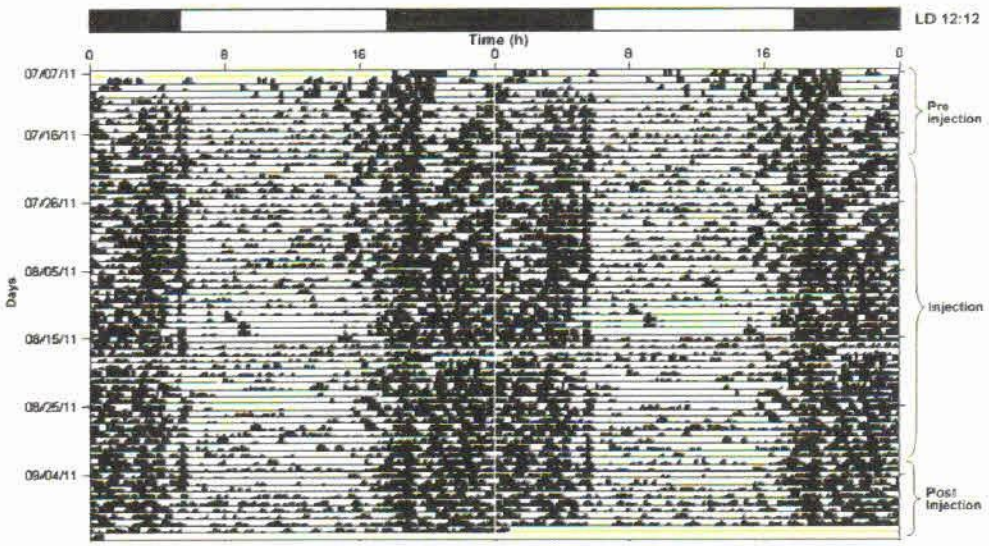


Figure 21.b : AC treated rats

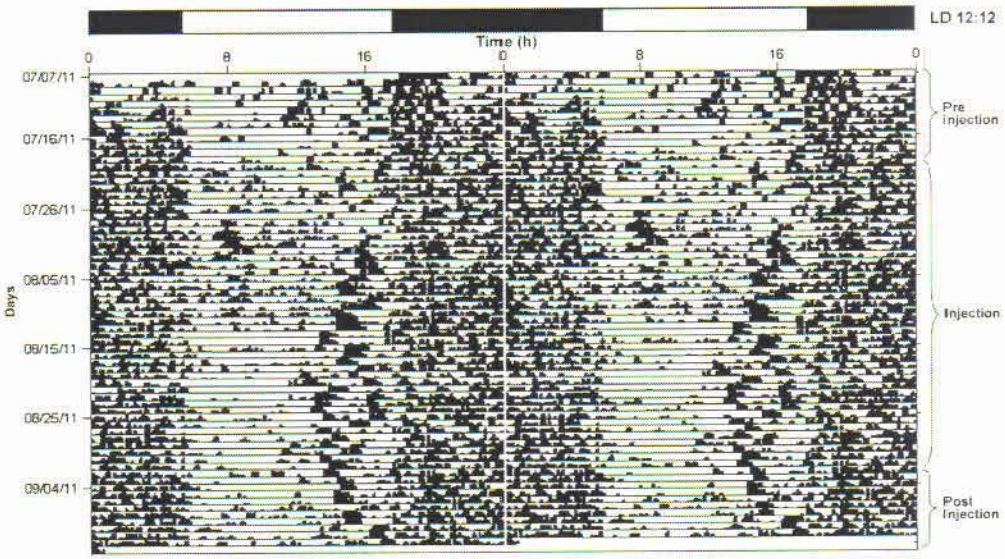


Figure 21.c : AC + fisetin treated rats

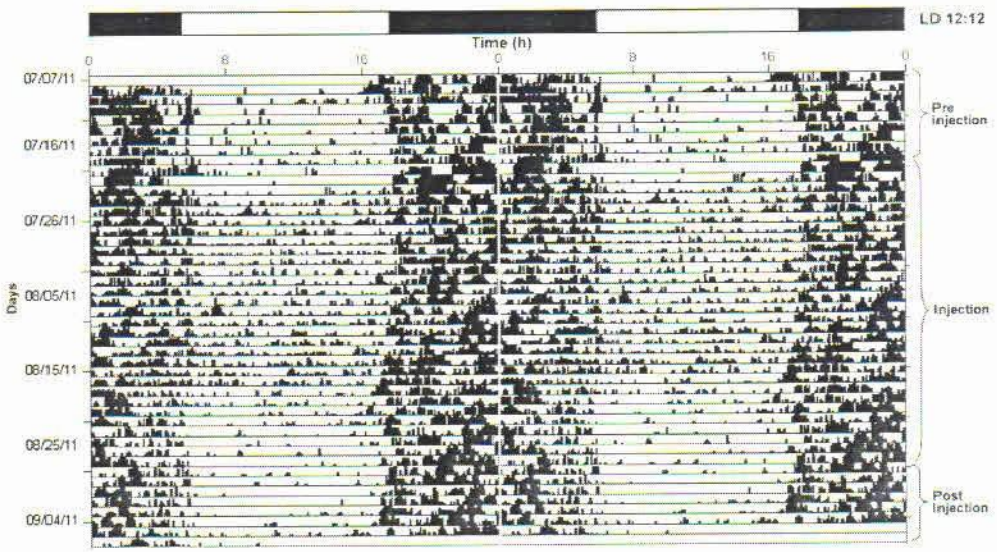


Figure 21.d : Fisetin treated rats

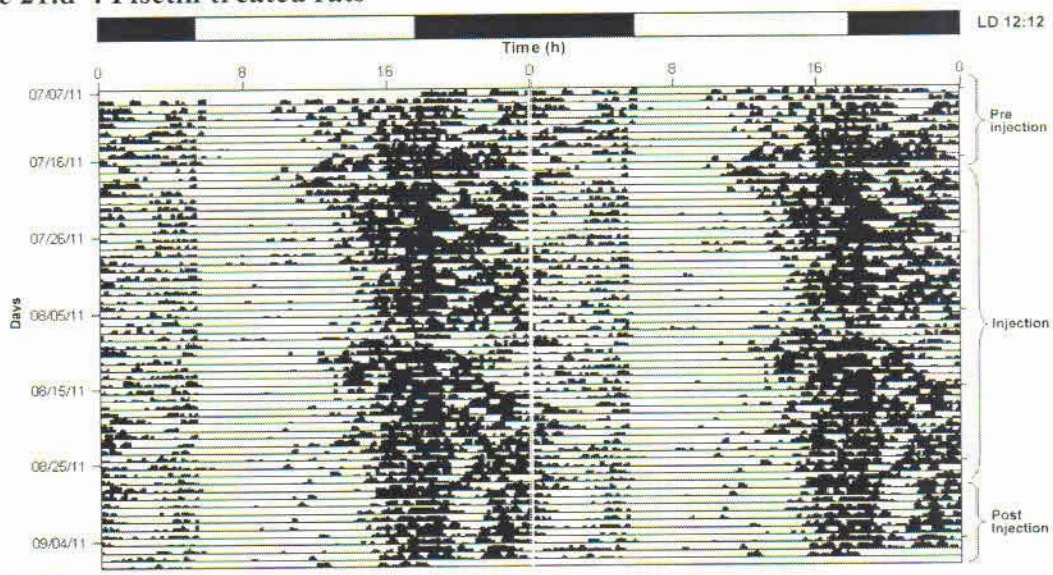


Table 1: Period changes in each group

Sl No:	Groups	Pre-Injection	Injection	Post-Injection
1.	Control	24.17	24.02	23.56
2.	AC	24.07	24.52	23.13
3.	AC+Fisetin	24.10	24.30	23.43
4.	Fisetin	24.12	23.58	24.08

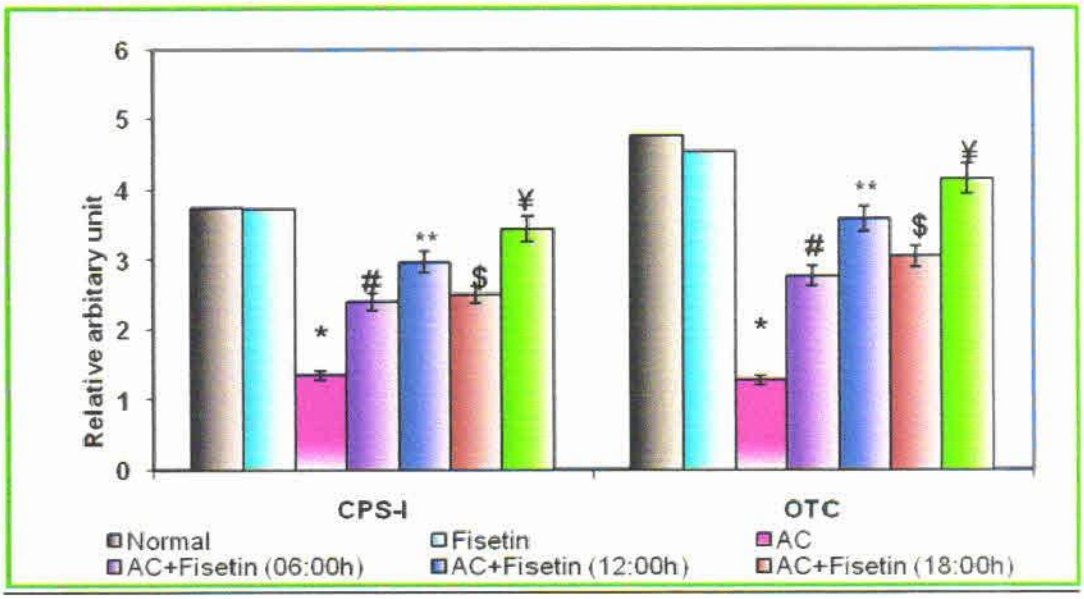
Table 2: Body weight changes in each group

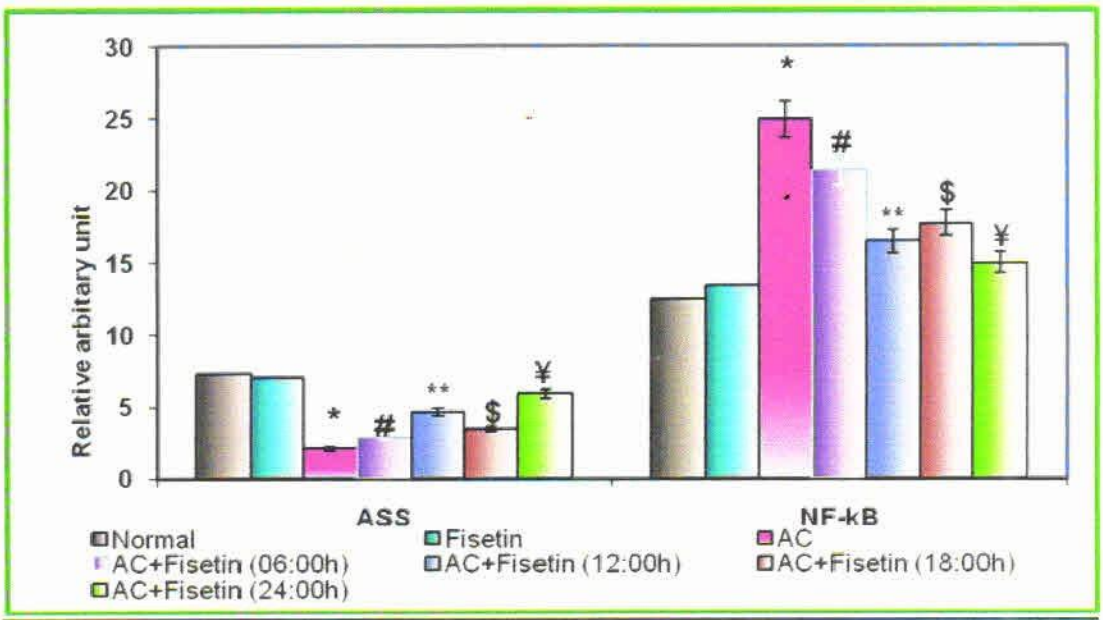
(Values are mean ± SD from 6 rats in each group)

S.No.	Groups	Initial Body weight (g)	Final Body weight (g)
1.	Control	183.6 ± 12.3	356.4 ± 16.6^{a}
2.	AC (100 mg/kg)	185.4 ± 13.5	275.1 ± 11.3^{c}
3.	AC+Fisetin	186.1 ± 13.9	321.7 ± 14.8^{b}
4.	Fisetin (50 mg/kg)	182.9 ± 11.7	350.6 ± 16.5^{a}

Data are presented as mean ± SD of 6 rats in each group. The values with different superscript (a, b, c) are significant from each others. $p<0.05$ mean values are significantly different from the other groups (ANOVA followed by DMRT)

Figure 22.b) Band intensities scanned by densitometer





Histograms from densitometric analysis expressed as arbitary units and given as mean ±SD of their observations.

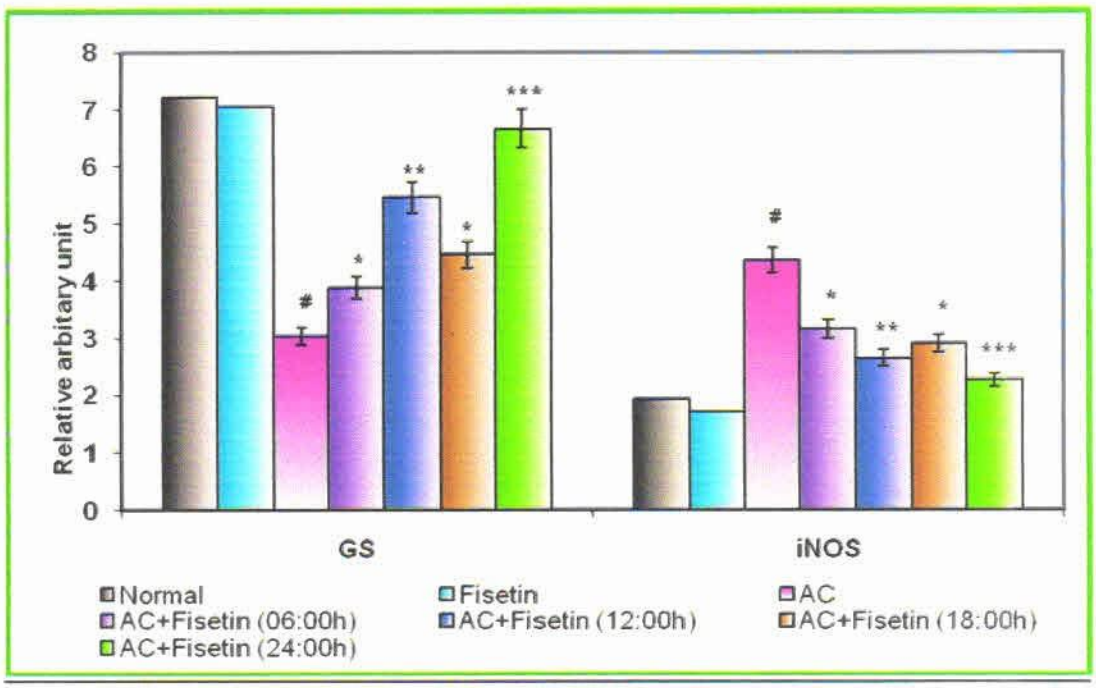
* - P<0.05 compared with control rats.

ψ - P<0.05 compared with AC Treated rats

** - P<0.05 compared with AC Treated rats

\# - P<0.05 compared with AC Treated rats

$ - P<0.05 compared with AC Treated rats

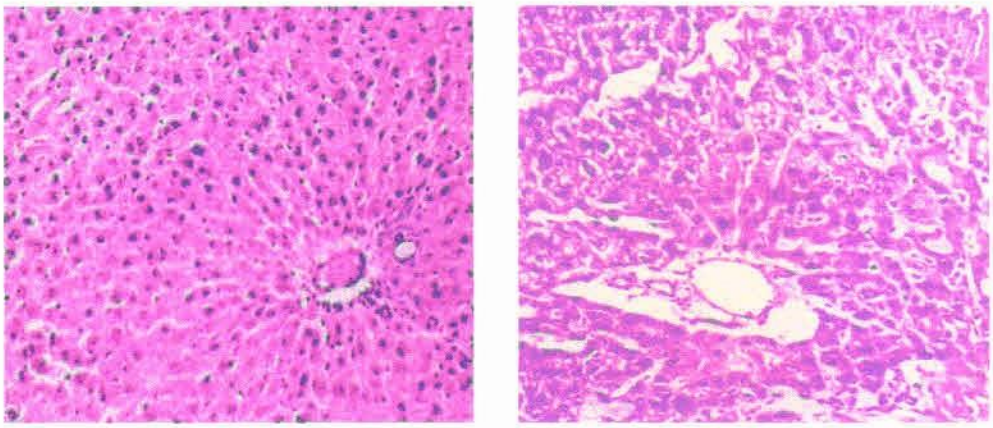


Histograms from densitometric analysis expressed as arbitary units and given as mean ±SD of their observations.

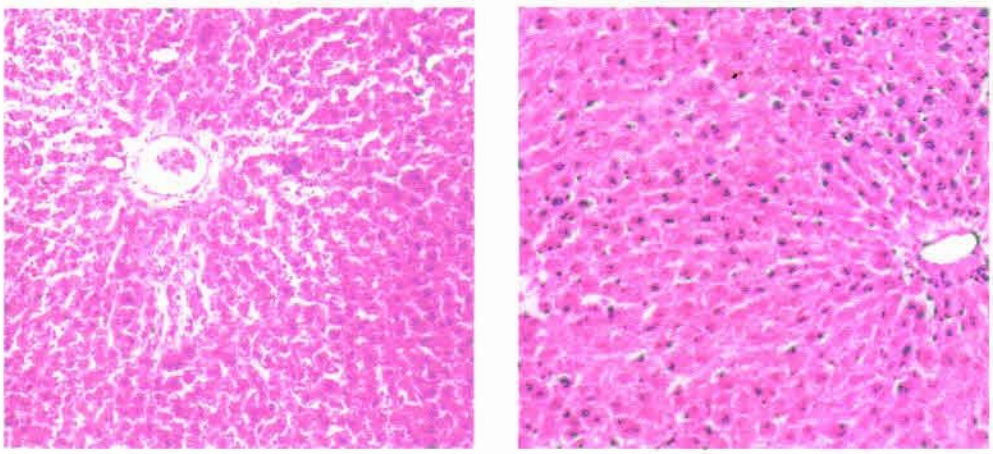
* - P<0.05 compared with control rats.
ψ - P<0.05 compared with AC Treated rats
** - P<0.05 compared with AC Treated rats
# - P<0.05 compared with AC Treated rats
$ - P<0.05 compared with AC Treated rats

Histopathology of liver

Figure 23. **A) Control** **B) AC treated**



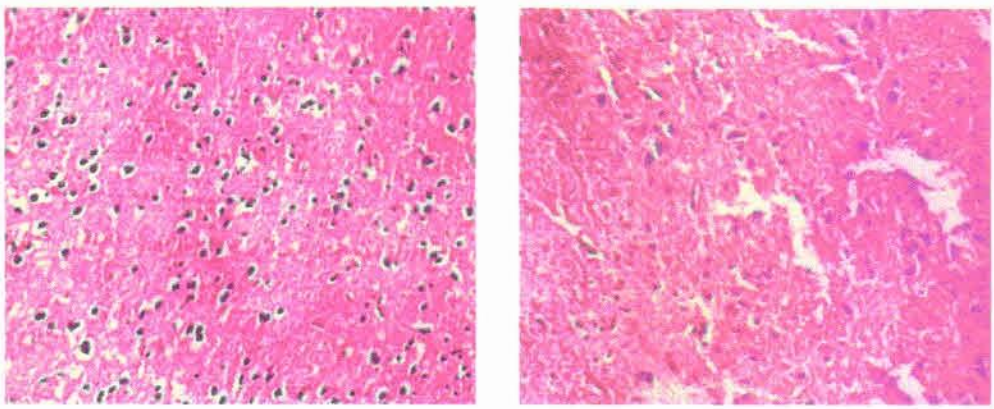
C) AC+Fisetin **D) Fisetin**



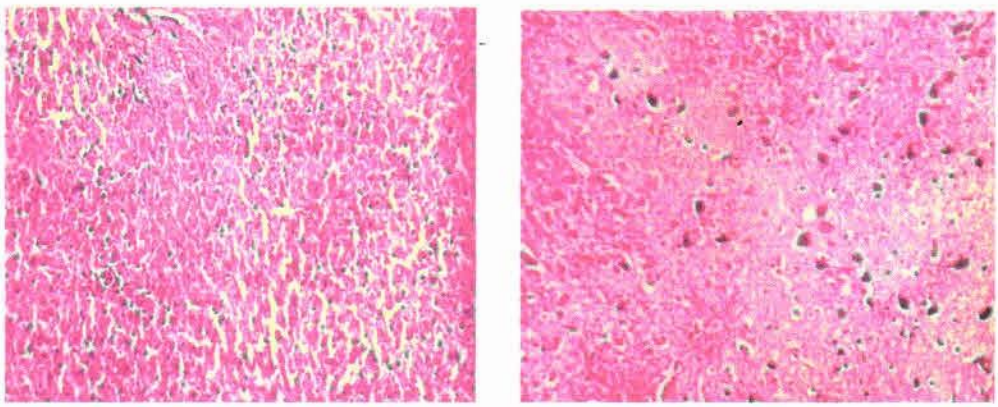
A) Control rat liver showing essentially normal liver architecture with hepatocytes radiating form the central vein and sinusoids. **B)** Section of AC treated rat liver showing the gross distortion of the normal architecture, prominence and widening of sinusoids. **C)** AC+Fisetin treated rat liver showing the ameleoration of distorted architecture. **D)** Section of fisetin treated rat liver showing the normal architexture. (All figures are of H&E-stained sections)

Histopathology of brain

Figure 24. **A) control** **B) AC treated**



C) AC+Fisetin **D) Fisetin**



A) Control rat brain showing the typical normal appearance of the cerebral cortex. **B)** Section of the AC treated rat brain showing disorganization of the typical appearance of the cerebral cortex. **C)** AC+Fisetin treated rat brain showing the ameleoration of disorganized architecture. **D)** Section of fisetin treated rat brain showing the normal architexture. (All figures are of H&E-stained sections)

ज्ञान-विज्ञान विमुक्तये

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI – 110 002.

STATEMENT OF EXPENDITURE IN RESPECT OF MAJOR/MINOR RESEARCH PROJECT

1. Name of Principal Investigator : **Dr. P. SUBRAMANIAN**
   Professor
2. Dept. of University/College : Dept. of Biochemistry & Biotechnology
   Faculty of Science, Annamalai
   University, Annamalai nagar - 608002
   Tamil nadu - India
3. UGC approval No. and Date : 37-46/2009 (SR) dated 12.01.2010
4. Title of the Research Project : "Chronopharmacological and Chronotherapeutic effect of fisetin in Hyperammonemic rats"
5. Effective date of starting the project : **01.02.2010**
6. (a) Period of Expenditure : From **01.02.2010** to **31.05.2013**
   (b) Details of Expenditure :

S. No.	Item	Amount Approved (Rs.)	Amount Released as I & II Instalments (Rs.)	Expenditure Incurred (Rs.)	Committed Expenses (Rs.)
1.	Books and Journals	-	7,49,800.00 & 5,35,506.00	-	-
2.	Equipment	2,50,000.00		2,50,000.00	-
3.	Contingency	45,000.00		45,000.00	-
4.	Field work/ Travel	-		-	-
5.	Hiring Services	-			
6.	Chemicals and Glassware	5,00,000.00		5,00,000.00	-
7.	Overhead	83,300.00		83,300.00	-
8.	**Any other items** – Project Fellow (With revised payment scale)	5,12,785.00		4,05,486.00	1,05,779.00
	Total :	**13,91,085.00**	**12,85,306.00**	**12,83,786.00**	**1,05,779.00**

Total Grand Approved for Three years : Rs. **13,91,085.00/-**
Total Grand Received for Three years : Rs. **12,85,306.00/-**
Expenditure incurred : Rs. **12,83,786.00/-**
Committed expenses to be released : Rs. **1,05,779.00/-**
Unspent Amount : Rs. **1,520.00/-**

Staff :

Date of appointment : 10.02.2010

ITEMS	From to	Amount approved (Rs.)	Grand released (Rs.)	Expenditure incurred (Rs.)	Committed expenses (Rs.)
Project Fellow Salary @ Rs.14,000/- p.m. for first two years and Rs.16,000/-p.m. for third year as per revised scheme effective from 01.04.2010	10. 02. 2010 to 31. 01. 2013	**5,12,785.00/-**	**4,07,006.00/-**	**4,05,486.00/-**	**1,05,779.00/-**

1. It is certified that the appointment (s) have been made in accordance with the terms and conditions laid down by the commission.

2. If as a result of check or audit objective, some irregularity noticed, at a later date , action will be taken to refund, adjust or regularized the object amounts.

3. It is certified that the grant of **Rs. 12,85,306.00/-** (Twelve Lakhs Eighty five thousand Three Hundred and six only) received from the University Grants Commission under the scheme of support for Major Research Project entitled **"Chronopharmacological and Chronotherapeutic effect of fisetin in Hyperammonemic rats"** vide F.No. **37-46/2009 (SR) dated 12.01.2010**. The amount **Rs. 12,85,306.00/-** has been fully utilized for the purpose for which it was sanctioned and in accordance with the terms and conditions laid down by the University Grants Commission.

31.03.2013

SIGNATURE OF THE PRINCIPAL INVESTIGATOR

Dr. P. SUBRAMANIAN
PRINCIPAL INVESTIGATOR
UGC PROJECT
DEPARTMENT OF BIOCHEMISTRY
FACULTY OF SCIENCE
ANNAMALAI UNIVERSITY
ANNAMALAINAGAR 608 002

6/6/13

REGISTRAR SIGNATURE WITH OFFICE SEAL

REGISTRAR
ANNAMALAI UNIVERSITY

ज्ञान-विज्ञान विमुक्तये

Annexure-IV

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI-110 002

UTILIZATION CERTIFICATE

Certified that the grant of **Rs. 12,85,306.00/-** (Rupees **Twelve Lakhs Eighty five thousand Three Hundred and six** only) received from the University Grants Commission under the scheme of support for Major Research Project entitled "**Chronopharmacological and chronotherapeutic effect of fisetin in hyperammonemic rats**" vide UGC letter No. 37-46/2009(SR) dated 12.01.2010. The amount of **Rs. 12,85,306.00/-** (Rupees **Twelve Lakhs Eighty five thousand Three Hundred and six** only) has been utilized for the purpose for which it was sanctioned and in accordance with the terms and conditions laid down by the University Grants Commission. So I request you to kindly sanction the **committed expenses** of the above project.

Total Amount Allocated	: Rs. **13,91,085.00**/-
Total Grant Received	: Rs. **12,85,306.00**/-
Total Grant Utilized	: Rs. **12,83,786.00**/-
Committed Expenditure	: Rs. **1,05,779.00**/-

I kindly request you to sanction the committed expenses **Rs. 1,05,779.00**/- [From the allocated amount of **Rs. 13,91,085.00/-**]

31.03.2013

SIGNATURE OF THE PRINCIPAL INVESTIGATOR

Dr. P. SUBRAMANIAN
PRINCIPAL INVESTIGATOR
UGC PROJECT
DEPARTMENT OF BIOCHEMISTRY
FACULTY OF SCIENCE
ANNAMALAI UNIVERSITY
ANNAMALAINAGAR 608 002

SIGNATURE OF THE FINANCIAL OFFICER

FINANCE OFFICER
ANNAMALAI UNIVERSITY
ANNAMALAINAGAR 608 002

SIGNATURE OF THE REGISTRAR

REGISTRAR
ANNAMALAI UNIVERSITY

Biological Rhythm Research
2012, 1–12, i*First* article

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Chronotherapeutic influence of fisetin on ammonium chloride-induced hyperammonemic rats

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Ammonia is a key neurotoxin involved in the neurological complications of the liver. Elevated ammonia leads to hyperammonemic condition which affects several important central nervous system functions. Fisetin is one of the naturally occurring flavonoids found in fruits and vegetables; it exhibits a wide variety of therapeutic benefits such as anticancer, antidiabetic, antioxidant, antiangiogenic, and neuroprotective effects. In this present study, chronotherapeutic efficacy of fisetin on ammonium chloride (AC)-induced hyperammonemic rats was aimed to establish the maximum drug effect by determining the best fitting biological time for drug dosing that helps to increase the therapeutic index of fisetin. The antihyperammonemic effect of fisetin was determined by administering (50 mg/kg b.w. oral) to rats at 06:00, 12:00, 18:00 and 24:00 h against AC (100 mg/kg b.w. i.p.) induced hyperammonemic Wistar rats (180-200 g). Amelioration of patho-physiological conditions of AC-induced hyperammonemia rats by fisetin at different time points (06:00, 12:00, 18:00 and 24:00 h) were measured by assessing the circulatory levels of ammonia, urea, uric acid, creatinine, bilirubin, and liver marker enzymes. Fisetin administration at 24:00 h showed more significant effects on those parameters than the other time points ($p<0.05$) and this might be due to pharmacokinetic property of the drug (fisetin) on temporal variations.

Keywords: hyperammonemia; fisetin; chronotherapy; flavonoids; ammonium chloride

Introduction

Degradation of proteins and other nitrogenated compounds produces ammonia; at high concentrations, ammonia is toxic, leading to impairment of cerebral function (Kojic et al. 2005). Ammonia is detoxified in ureotelic animals, detoxification occurs in the liver by incorporating ammonia into urea, a process carried out by the urea cycle. Ammonia detoxification is impaired in liver failure and in congenital deficiencies of the urea cycle enzymes (Nassogne et al. 2005; Enns et al. 2007). Chronic liver disease is an important cause of death in Western countries. When the liver fails or when blood is shunted past the liver (e.g. in liver cirrhosis), ammonia is not adequately detoxified, leading to hyperammonemia and hepatic encephalopathy with altered brain function. The signs of hepatic encephalopathy in patients with chronic liver disease range from alterations in the sleep–waking cycle and motor

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http://www.tandfonline.com

coordination to changes in personality, gradually developing intellectual impairment. Hepatic encephalopathy may lead to coma and death.

Hyperammonemia is considered as the main factor responsible for the alterations in cerebral function associated with hepatic encephalopathy (Felipo and Butterworth 2002; Monfort et al. 2009; Bosoi and Rose 2009; Anne et al. 2010). Acute ammonia intoxication leads to activation of N-methyl D-aspartic acid (NMDA) receptors in the brain allowing the entry of Ca^{2+} and Na^{+} in the postsynaptic neuron. Calcium is absorbed by mitochondria, resulting in mitochondrial calcium accumulation, impairment of mitochondrial respiration, decreased ATP synthesis, and increased formation of free radicals leading to oxidative stress. Cytosolic Ca^{2+} binds with calmodulin (CM) and activates both nitric oxide synthase (NOS) and calcineurin (CN). Activation of NOS increases the formation of nitric oxide (NO), which affects the activity or function of different enzymes and proteins both in neuronal mitochondria and cytosol and likely in neighboring astrocytes. The activation of CN leads to dephosphorylation of a residue of Na^{+}/K^{+} ATPase phosphorylated by protein kinase (PKC). This results in activation of the ATPase, which facilitates the removal of the excess of Na^{+} entering through the NMDA receptor, but increases the consumption of ATP. This, together with the reduced synthesis, leads to depletion of ATP. The disadvantages of presently available antihyperammonemic agents and therapies are sometimes inadequate and have serious adverse effects (Srinivasan et al. 2001). The screening and development of drugs for antihyperammonemic activity is still in progress. There is a need of search for appropriate protective agents against hyperammonemia. This can be achieved by focusing on active principles of plants because natural products that may offer better treatment than currently used drugs.

Flavonoids comprise a large group of plant secondary metabolites characterized by a diphenylpropane structure (C6-C3-C6). Flavonoids are low-molecular weight polyphenolic substances based on the flavan nucleus and they are distinguished by the carbon position around the molecule. The three phenolic rings are referred to as A, B, and C (or pyrane) rings. The biochemical activities of flavonoids and their metabolites depend on their chemical structure and the relative orientation of various moieties on the molecule. They are widely distributed throughout the plant kingdom and are commonly found in fruits, vegetables, and certain beverages (Lopez-Lazaro 2009). They have been reported to exhibit a wide range of biological effects, including antibacterial, antiviral, anti-inflammatory, antiallergic, and antioxidant (Cook and Samman 1996). Fisetin (3, 3′, 4′, 7-tetrahydroxyflavone) is a dietary flavonoid widely distributed in strawberries, apples, persimmons, grapes, onions, and cucumbers at concentrations of 2–160 μg/g (Arai et al. 2000) and displays a variety of pharmacological properties including antioxidant (Hanneken et al. 2006), antiallergic (Cheong et al. 1998), anti-inflammatory (Park et al. 2007), anticancer (Sung et al. 2007), neuroprotective (Zbarsky et al. 2005), neurotrophic (Maher et al. 2006), and antiangiogenic (Fotsis et al. 1998).

Mammalian circadian pacemaker resides in the paired suprachiasmatic nuclei (SCN) and influences a multitude of biological processes. Clock genes are the genes that control the circadian rhythms in physiology and behavior. Twenty-four-hour rhythm is demonstrated for the function of physiology and the pathophysiology of diseases. The effectiveness and toxicity of many drugs vary depending on dosing time. Such chronopharmacological phenomena are influenced by not only the pharmacodynamics but also pharmacokinetics of medications. The underlying

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mechanisms are associated with 24-h rhythms of biochemical, physiological, and behavioral processes under the control of circadian clock (Lala and Nandgaonkar 2010). Thus, the knowledge of 24-h rhythm in the risk of disease and evidence of 24-h rhythm dependencies of drug pharmacokinetics, effects, and safety constitutes the rationale for pharmacotherapy. Chronotherapy is especially relevant, when the risk and/or intensity of the symptoms of disease vary predictably over time as exemplified by cancer (Takimoto 2006; Levi et al. 2007), allergic rhinitis and asthma (Smolensky et al. 2007), arthritis, myocardial infarction, hypertension (Smolensky and Haus 2001; Hermida et al. 2007), congestive heart failure, stroke (Otsuka et al. 2003), peptic ulcer, and sleep disorders (Pandi-Perumal et al. 2008).

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Materials and methods

Experimental animals

Healthy male albino Wistar rats (180–200 g) were obtained from the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College and Hospital, Annamalai University, and maintained in an air-conditioned room ($25 \pm 3^{\circ}C$) with a 12 h light/12 h dark cycle. Feed and water were provided *ad libitum* to all the animals. The study protocols were approved by the Institutional Animal Ethics Committee of Rajah Muthiah Medical College and Hospital (Reg No.160/1999/CPCSEA, Approval No: 737, 2 September 2010), Annamalainagar.

Chemicals

Fisetin was purchased from Shanxi Jintāi Biological (China). Ammonium chloride and other chemicals used in this study were of analytical grade obtained from E. Merck and HIMEDIA, India.

Experimental induction of hyperammonemia

Hyperammonemia was induced in Wistar rats by intraperitoneal injections of a freshly prepared solution of ammonium chloride at a dose of 100 mg/kg body weight thrice in a week for eight consecutive weeks (Subash et al. 2007).

Experimental design

The rats were randomly divided into seven groups with six rats in each group as given below. Fisetin was completely dissolved in 0.5% DMSO.

Group 1: Control rats orally administered with 0.5% DMSO
Group 2: Rats orally administered with fisetin (50 mg/kg b.w.) (Shia et al. 2009)
Group 3: Rats treated with AC (100 mg/kg b.w, i.p injections)
Group 4: Rats treated with AC+ Fisetinto be administered at 06:00 h
Group 5: Rats treated with AC+ Fisetinto be administered at 12:00 h
Group 6: Rats treated with AC+ Fisetinto be administered at 18:00 h
Group 7: Rats treated with AC+ Fisetinto be administered at 24:00 h

At the end of the experimental period (eighth week), all animals were made to fast overnight and were sacrificed by cervical dislocation.

Serum preparation

Blood was collected in a dry test tube and allowed to coagulate at ambient temperature for 30 min. Serum was separated by centrifugation at 2000 rpm for 10 min.

Preparation of plasma

The blood, collected in a heparinized centrifuge tube, was centrifuged at 2000 rpm for 10 min and the plasma was separated by aspiration. The separated plasma was used for estimations.

Biochemical estimations

Blood ammonia was determined by enzymatic colorimetric assay of Wolheim (1984), which was performed using automated Roche/Hitachi 912 kit. To 20 μl of the blood, 200 μl of triethanolamine and 150 μl of NADPH/GLDH/buffered substrate were added, mixed well, and the absorbance was read at 470 nm.

Plasma urea was determined by diacetylmonoxime method (Varley et al. 1998), which was performed using automated Roche/Hitachi 912 kit. To 0.1 ml of plasma, 3.3 ml of water was added and mixed. Then 0.3 ml of 10% sodium tungstate and 0.3 ml of 0.67 N sulfuric acid were added, mixed and centrifuged. To 2.0 ml of the supernatant, 2.0 ml of water, 0.4 ml of diacetylmonoxime, and 1.6 ml of sulfuric acid–phosphoric acid mixture were added and heated in a boiling water bath for 30 min and cooled. Different aliquots of standards were taken and treated in the same manner with the blank and were read at 480 nm.

Uric acid was usually determined by Caraway method (Varley et al. 1998). 5.4 ml of diluted tungstic acid was added to 0.6 ml of plasma. The contents were mixed and centrifuged. In to the test tubes, 3 ml of supernatant, standard and water (as blank) were taken, 0.6 ml of sodium carbonate and 0.6 ml of phosphotungstic acid reagent were added, mixed and placed in a 25°C water bath for 10 min. The blue color developed was read at 700 nm.

Serum creatinine was determined by the method of Slot (Varleyet al., 1998), which was performed using automated Roche/Hitachi 912 kit. To 3 ml of deproteinized supernatant (0.1 ml of serum + 3.9 ml of 10% TCA), 2 ml of alkaline picrate solution was added. Blank containing 3 ml of water and aliquots of standard in 3 ml of water were also treated in a similar manner. After 30 min, the color was read at 520 nm against the reagent blank.

Serum bilirubin was estimated by the method of Malloy and Evelyn, (1937) by Van den Bergh reaction. It was based on the formation of purple colored azobilirubin when bilirubin reacts with diazotized sulphanilic acid. 0.2 ml of serum was diluted to 2.0 ml with distilled water in two tubes marked as test and blank. To the test, 0.5 ml of the diazo reagent and to the blank, 0.5 ml of 1.5 % hydrochloric acid was added. Finally to both tubes, 2.5 ml of methanol was added and the tubes were kept at room temperature for 30 min. The color developed was read at 540 nm.

Estimation of liver marker enzymes

Activities of AST and ALT were assayed by the method of Reitman and Frankel (1957). 0.2 ml aliquot of serum with 1 ml of substrate (aspartate and α-ketoglutarate

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(KG) for AST: alanine and α-KG for ALT) in phosphate buffer (pH 7.4) was incubated for 1 h for AST and 30 min for ALT. 1 ml aliquot of 2,4-dinitrophenylhydrazine (DNPH) solution was added to arrest the reaction and was incubated for 20 min at 25°C. After incubation, 1 ml of 0.4 N NaOH was added and the absorbance was read at 540 nm. Activities are expressed as IU/L.

Alkaline phosphatase (ALP) was assayed by the method of King and Armstrong (1934). The ALP activity was assayed using disodium phenylphosphate as substrate. After preincubation of the buffer (0.1 M bicarbonate buffer, pH 10) with the substrate for 10 min, 0.2 ml of serum was added and incubated for 15 min at 25°C. The liberated phenols from the substrate reacted with folin–phenol reagent (1 ml). The suspension was centrifuged and collected as the supernatant. Aliquot of 10% sodium bicarbonate 2 ml was added to the supernatant and the color that developed was read at 680 nm after 10 min. Activities of ALP are expressed as IU/L. Gamma-glutamyl transferase (GGT) activity was determined by the method of Fiala et al. (1972).

Statistical analysis

Statistical analysis was performed by one way Analysis of Variance (ANOVA) followed by Duncan's multiple range test (DMRT) using Software Package for the Social Science (SPSS) software package version 12.00. Results were expressed as mean ± SD for six rats in each group. p values <0.05 were considered significant.

Results

The levels of blood ammonia, plasma urea, uric acid, serum creatinine, and bilirubin in control and experimental rats are shown in Figures 1–5. The levels of circulatory ammonia, urea, uric acid, serum creatinine, and bilirubin were significantly higher in AC-treated rats when compared with control rats. Hyperammonaemic rats treated with fisetin at different time points significantly normalized the levels of ammonia, urea, uric acid, creatinine, and bilirubin, as compared with hyperammonaemic rats (Tables 1 and 2). The levels of circulatory liver marker enzymes in control and

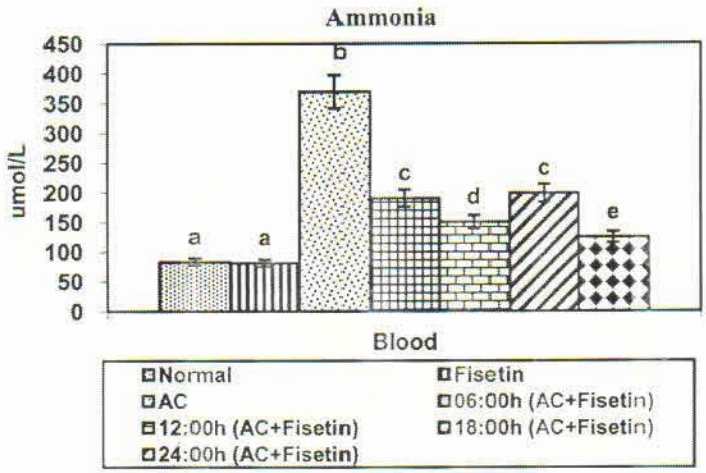

Figure 1. Chronotherapeutic effect of fisetin on blood ammonia of normal and experimental rats. Values are expressed as means ± SD for six rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).

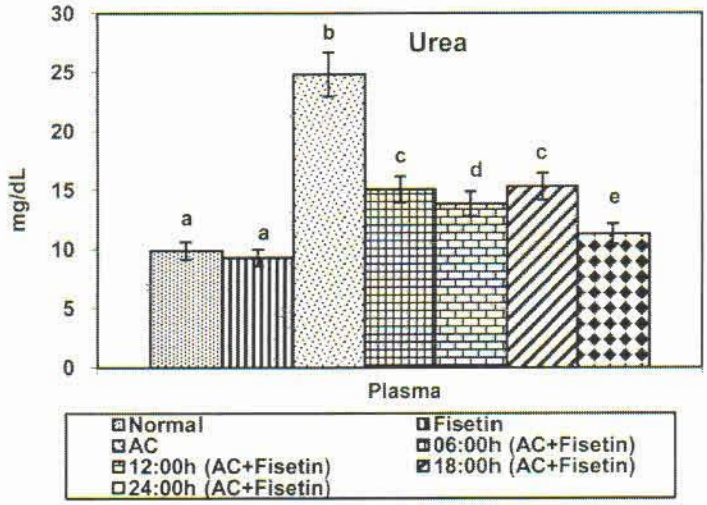

Figure 2. Chronotherapeutic effect of fisetin on plasma urea of normal and experimental rats. Values are expressed as means ± SD for six rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).

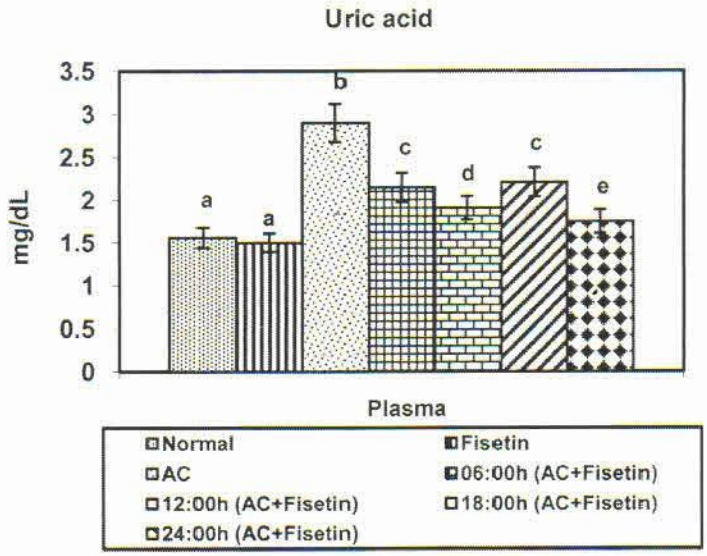

Figure 3. Chronotherapeutic effect of fisetin on plasma uric acid of normal and experimental rats. Values are expressed as means ± SD for six rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).

experimental groups are given in Figures 6–9. The level of liver marker enzymes significantly increased in AC-treated rats, and these levels were significantly normalized (Table 3) in hyperammonaemic rats treated with fisetin at different time points.

Discussion

The urea cycle is the metabolic pathway that eliminates excess nitrogen of the body by detoxification of ammonia into urea (Nassogne et al. 2005). Urea cycle disorders (UCDs) are inborn errors of metabolism caused by a deficiency of any of the

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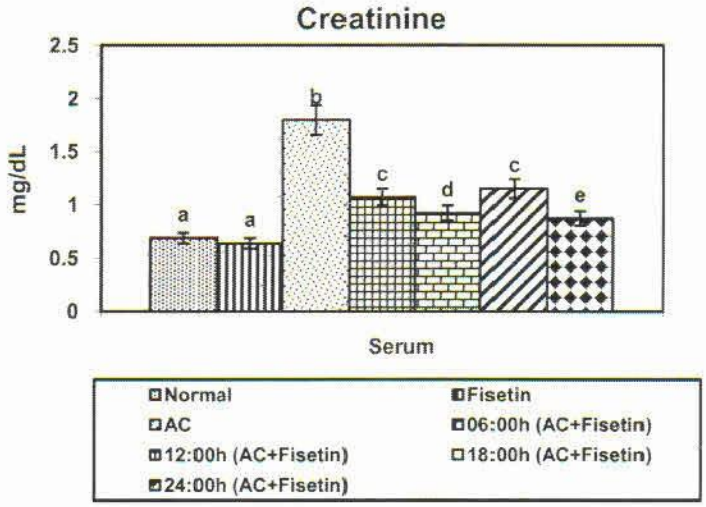

Figure 4. Chronotherapeutic effect of fisetin on changes in the serum creatinine of normal and experimental rats. Values are expressed as means ± SD for six rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).

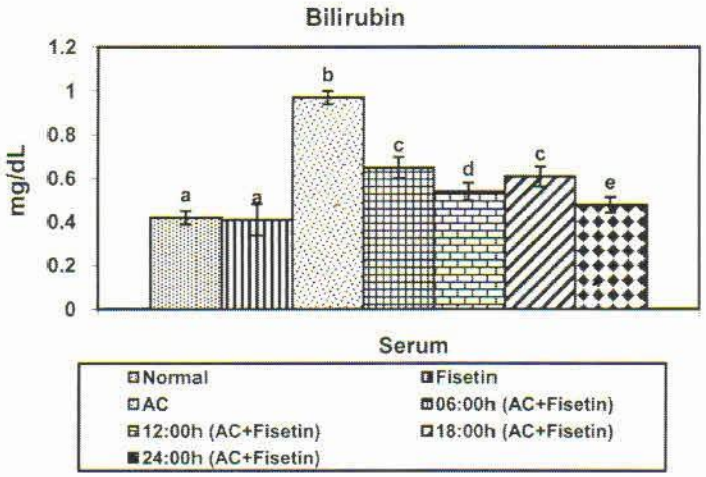

Figure 5. Chronotherapeutic effect of fisetin on serum bilirubin of normal and experimental rats. Values are expressed as means ± SD for six rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).

Table 1. Chronotherapeutic effect of fisetin on changes in blood ammonia, plasma urea and uric acid of normal and experimental rats.

Groups	Ammonia (μmol/L)	Urea (mg/dl)	Uric acid (mg/dl)
Normal	83.70 ± 6.23^{a}	9.87 ± 0.74^{a}	1.56 ± 0.12^{a}
Fisetin (50 mg/kg)	81.94 ± 6.14^{a}	9.28 ± 0.70^{a}	1.50 ± 0.11^{a}
AC treated (100 mg/kg)	369.48 ± 27.71^{b}	24.81 ± 1.86^{b}	2.90 ± 0.22^{b}
AC + Fisetin (06:00 h)	191.06 ± 14.33^{c}	15.03 ± 1.13^{c}	2.15 ± 0.17^{c}
AC + Fisetin (12:00 h)	150.86 ± 11.32^{d}	13.81 ± 1.04^{d}	1.91 ± 0.14^{d}
AC + Fisetin (18:00 h)	199.46 ± 14.96^{c}	15.28 ± 1.15^{c}	2.21 ± 0.17^{c}
AC + Fisetin (24:00 h)	125.46 ± 9.41^{e}	11.25 ± 0.85^{e}	1.75 ± 0.14^{e}

Note: Values are given as mean ± S.D from eight rats in each group. Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT).

Table 2. Chronotherapeutic effect of fisetin on changes in serum creatinine and bilirubin of normal and experimental rats.

Groups	Creatinine (mg/dl)	Bilirubin (mg/dl)
Normal	0.69 ± 0.05^{a}	0.42 ± 0.031^{a}
Fisetin (50 mg/kg)	0.64 ± 0.05^{a}	0.41 ± 0.030^{a}
AC treated (100 mg/kg)	1.80 ± 0.14^{b}	0.97 ± 0.072^{b}
AC +Fisetin (06:00 h)	1.07 ± 0.08^{c}	0.65 ± 0.048^{c}
AC+ Fisetin (12:00 h)	0.92 ± 0.07^{d}	0.54 ± 0.041^{d}
AC+ Fisetin (18:00 h)	1.15 ± 0.09^{c}	0.61 ± 0.046^{c}
AC+ Fisetin (24:00 h)	0.87 ± 0.07^{e}	0.48 ± 0.036^{e}

Note: Values are given as mean ± SD from eight rats in each group. Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT).

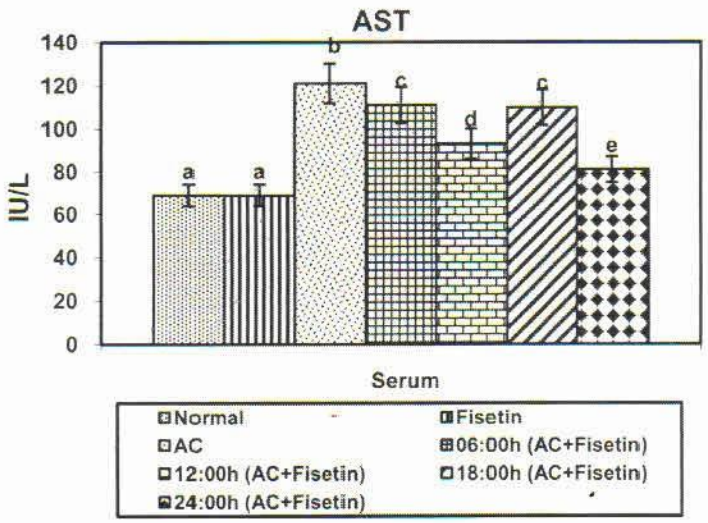

Figure 6. Chronotherapeutic effect of fisetin on serum AST of normal and experimental rats. Values are expressed as means ± SD for six rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).

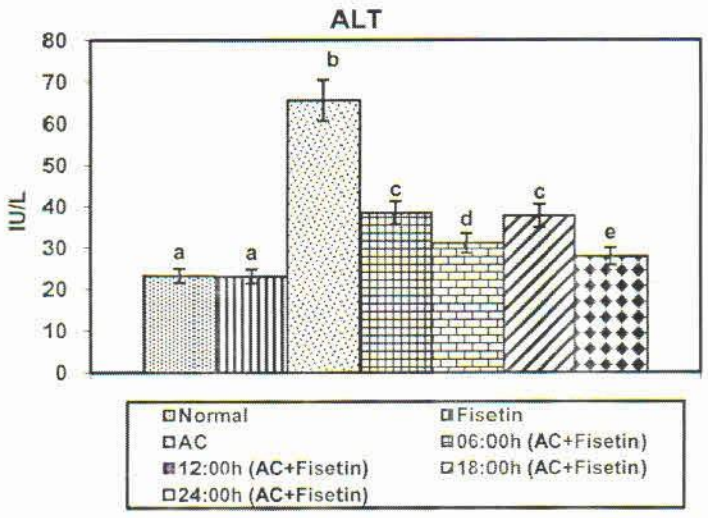

Figure 7. Chronotherapeutic effect of fisetin on serum ALT of normal and experimental rats. Values are expressed as means ± SD for six rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).

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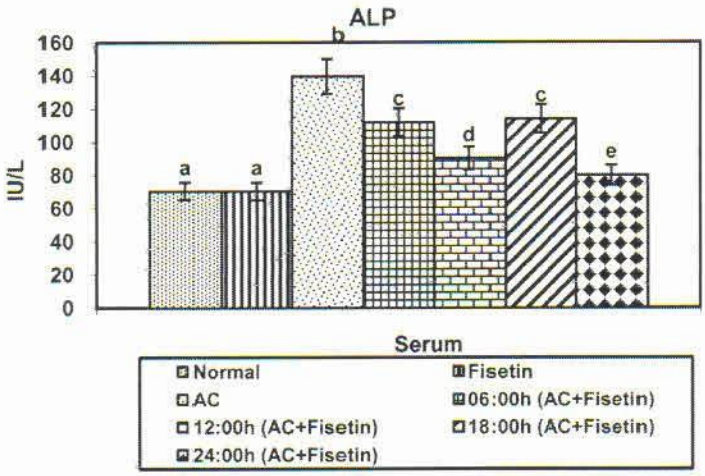

Figure 8. Chronotherapeutic effect of fisetin on serum ALP of normal and experimental rats. Values are expressed as means ± SD for six rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).

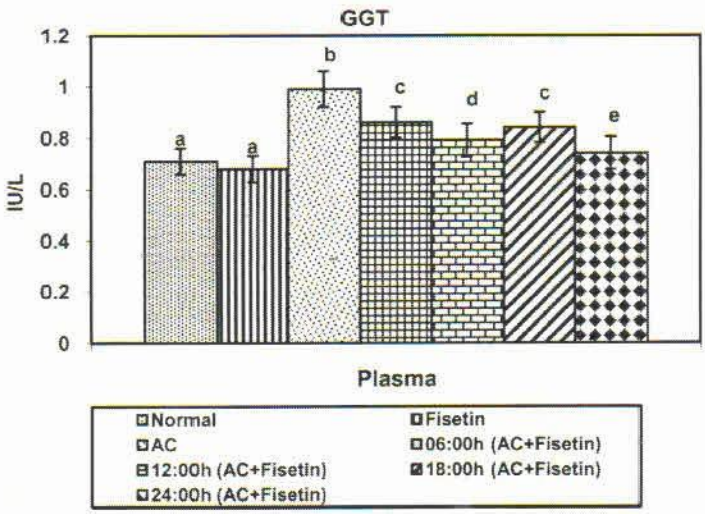

Figure 9. Chronotherapeutic effect of fisetin on serum GGT of normal and experimental rats. Values are expressed as means ± SD for six rats in each group. Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).

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enzymes that constitute the urea cycle. Elevation of blood ammonia results in hyperammonemia, it enhances the production of reactive species of nitrogen/oxygen leads to the oxidative stress mediated deleterious effects through several accumulation of glutamine, predominantly in the astrocytes, leading to hepatic encephalopathy and in congenital defects of ammonia detoxication (Felipo and Butterworth 2002).

Chronopharmacokinetics defines the temporal changes in absorption, distribution, metabolism, and elimination of a pharmacological agent. It describes the effect of time of administration. Almost every function in the kidney shows a 24-h rhythm, including glomerular filtration rate, renal blood flow, urinary pH, and proximal and distal tubular reabsorption. Several drugs have robust rhythms in renal excretion that can be accounted for by the time-dependent variability in the effect (Lala and

Table 3. Chronotherapeutic effect of fisetin on changes in serum AST, ALP, ALT and GGT in normal and experimental rats.

Groups	AST (IU/L)	ALP (IU/L)	ALT (IU/L)	GGT (IU/L)
Normal	68.98 ± 5.18^{a}	70.41 ± 5.28^{a}	23.21 ± 1.74^{a}	0.71 ± 0.05^{a}
Fisetin (50 mg/kg)	68.91 ± 5.17^{a}	70.32 ± 5.28^{a}	23.04 ± 1.72^{a}	0.68 ± 0.05^{a}
AC treated (100 mg/kg)	120.96 ± 9.07^{b}	139.56 ± 10.48^{b}	65.48 ± 4.91^{b}	0.99 ± 0.07^{b}
AC+ Fisetin (06:00 h)	110.97 ± 8.32^{c}	112.10 ± 8.40^{c}	38.39 ± 2.88^{c}	0.86 ± 0.06^{c}
AC+ Fisetin (12:00 h)	93.11 ± 6.98^{d}	90.27 ± 6.78^{d}	31.10 ± 2.34^{d}	0.79 ± 0.064^{d}
AC+ Fisetin (18:00 h)	109.86 ± 8.18^{c}	114.15 ± 8.57^{e}	37.67 ± 2.83^{c}	0.84 ± 0.06^{c}
AC+ Fisetin (24:00 h)	81.06 ± 6.08^{e}	80.15 ± 6.02^{e}	27.90 ± 2.10^{c}	0.74 ± 0.063^{e}

Note: Values are given as mean $\pm$ S.D from eight rats in each group. Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT).

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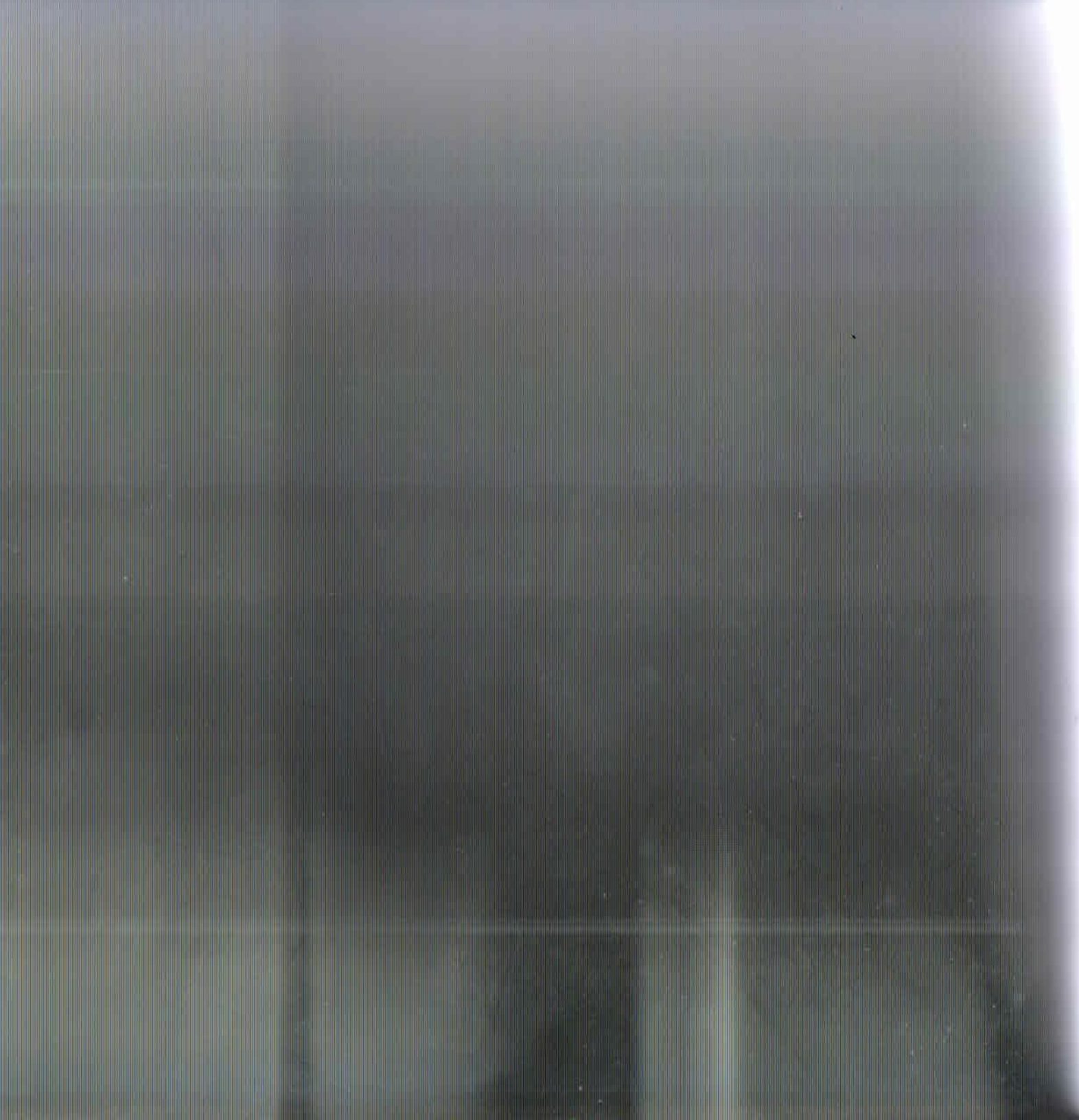
Nandgaonkar 2010). The urea concentration and the total urea content of the urine were higher during the night than during the day-time. Consistent with these findings, the urea concentrations in the liver and serum had circadian rhythms with the highest values at 02:00 h and the lowest values at 14:00 h. Argininosuccinate synthetase is considered as the rate limiting enzyme to play a critical role in regulation of urea formation, and thus it seems likely that the circadian rhythm of change in urea concentration might be caused by periodical change in the synthetase activity (Kato et al. 1978).

Our results reveal that the rhythms in animals are not synchronized/exhibit a phasing with that of normal rats. This lack of synchronization reflect as an alteration of circadian clock in hyperammonemic rats and may require specific measure for chronotherapy to improve therapeutic index. Increased levels of circulatory ammonia, urea, uric acid, and creatinine might indicate hyperammonaemic condition in rats treated with AC (Subash and Subramanian 2008), which may be due to liver damage caused by ammonia intoxication. Administration of fisetin to AC-induced hyperammonaemic rats at different time points significantly decreased the levels of blood ammonia and urea. Group 7 rats (24 h) showed decreased levels of ammonia, urea, uric acid and creatinine when compared with other time points. The reduction in the levels of ammonia and urea during fisetin treatment showed the potent anti-hyperammonaemic effect of fisetin. In fact, the pattern of the urea cycle enzymes rhythm coincided well with those of the circadian rhythms of changes in urea concentration in liver and serum.

Determination of activities of serum enzymes like AST, ALT, ALP and GGT can make assessment of liver function. When liver cell plasma membrane is damaged a variety of enzymes normally located in the cytosol are released in to the blood stream and their determination in the serum is a useful quantitative marker of the extent and type of hepatocellular damage (Balamurugan and Muthusamy 2008). Determination of serum bilirubin represents as an index for the assessment of hepatic function, and any abnormal increase in the levels of serum bilirubin indicate hepatobiliary disease and severe disturbances of hepatocellular function (Martin and Friedman 1992). Stabilization in the levels of serum bilirubun in the fisetin-treated groups as compared to AC-induced groups clearly indicates the improvement in the function status of liver.

Fisetin administration at 24:00 h showed significant effects on those parameters than the other time points, and it is due to pharmacological and pharmacokinetic



property of the drug on circadian variations of physiology of liver diseases and increased pH, renal clearance, expression and activity of urea cycle enzymes improves the tolerance and efficacy of the drug.

Conclusion

These findings showed that the naturally occurring compounds of plant origin ameliorate effectively the hyperammonemic condition by temporally. The beneficial chronotherapeutic effect of fisetin on ammonia detoxification offers exciting opportunity to develop them into novel therapeutics and enormous scope for combating the threat of hyperammonemia.

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