Biological Activity of *Chrysanthemum coronarium* L. Extracts

Abd El Raheim M. Donia[1,2*]

[1]*Pharmacognosy Department, College of Pharmacy, Salman Bin Abdulaziz University, Al-Kharj, Saudi Arabia.*
[2]*Department of Medicinal and Aromatic Plants, Desert Research Center, Cairo, Egypt.*

Author's contribution

This whole work was carried out by author A M Donia.

ABSTRACT

Aim: The current research was designed to evaluate the biological and antioxidant activities of *Chrysanthemum coronarium*.
Methodology: Two main experiments were designed to evaluate the biological activity of ethanolic extract *Chrysanthemum coronarium* first one concerned with hepatoprotective activity at concentrations of 200 and 400mg/kg, the second experiment was established to study the effect of the same doses on male fertility.
Results: Our results revealed that; total extract of *C. coronarium* and their four fractions (diethyl ether, chloroform, ethyl acetate and n-butanol) showed antioxidant activity, in which the highest activity achieved with ethyl acetate fraction. In biological evaluation *C. coronarium* at dose of 400mg/kg showed hepatoprotective activity, also it showed significant improvement of the relative weight of reproductive organs, sperm count, sperm motility, total sperm abnormality, testosterone and LH.
Conclusion: The results suggest that *C. coronarium* has a significant hepatoprotective activity and improve fertility at 400mg/kg.

Keywords: Chrysanthemum coronarium; antioxidant; hepatoprotective activity; sex hormones.

Corresponding author: Email: a.donia@sau.edu.sa; donia22276@yahoo.com;

1. INTRODUCTION

Chrysanthemum coronarium L. (Garland) is an annual herbaceous weed from the Asteraceae family native to the Mediterranean regions [1]. Phytochemical investigations of some members of the genus *Chrysanthemum* have revealed the presence of several odoriferous principles (cis-chrysanthenyl acetate, trans-chrysanthenyl acetate, α-pinene) and phenolic components (mainly quinic acid derivatives) [2]. Previous analyses of the essential oil of *C. coronarium* have indicated different compositional patterns owing to the origin and genotype. For example, the main components of the essential oil from Spanish specimens were camphor (29.2%), α-pinene (14.8%), lyratyl acetate (9.8%) [3]. In addition to its aromatic composition, *C. coronarium* known to be a rich source of phenolic components with a variety of biological activities [4]. Three quinic acid derivatives (chlorogenic acid, 3,5-dicaffeoylquinic acid and 4-succinyl-3,5-dicaffeoylquinic acid) with antioxidant activities in a comparative study on the phenolic constituents of *C. morifolium* and *C. coronarium* [2], chlorogenic acid, 1,5-dicaffeoylquinic acid, 1,3-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid and 4- succinyl-3,5-dicaffeoylquinic acid) were identified from *C. coronarium* [5]. HPLC–PDA-MS analysis allowed the identification of chlorogenic acid di-cafeoylquinic acids isomers, rutin, luteolin, luteolin-7-O-glucoside, myricetin-3-O-galactoside and tricin [6].

2. MATERIALS AND METHODS

2.1 Plant Material

Chrysanthemum coronarium was collected from Sidi Barani, North Western Coast, Egypt during spring (2009). The collected plant material was air-dried in shade, reduced to fine powder and kept dry for further studies.

2.2 Extraction

One kg of the dried powder of *C. coronarium* (aerial parts) was extracted by percolation in 70% aqueous ethanol (2011, pharmacognosy lab. Salman Bin Abdulaziz University, KSA)and this process repeated three times. Total ethanol extract was concentrated under reduced pressure at a temperature not exceeding 40°C to yield a dry extract of 145g. 50g of total ethanol extract was suspended in distilled water and extracted successively with diethyl ether, chloroform, ethyl acetate and n-butanol to give diethyl ether, chloroform, ethyl acetate and n-butanol fractions, respectively. Each fraction was dried over anhydrous sodium sulfate and the solvent was distilled off and kept at -4°C for further studies.

2.3 Antioxidant Activity

Antioxidant activity of total extract, diethyl ether, chloroform, ethyl acetate and n-butanol extracts was measured using 2-2-diphenyl-1-picrylhydrazyl (DPPH) and Trolox (2.5mM in methanol) as a reference substance. The presence of antioxidative substances in the assay leads to the reductive decoloration of the DPPH radical depending on the content of antioxidative substances 50µl of the sample was adjusted to 1mL with 50% methanol and then added to 1mL of DPPH reagent (7.5mg in 50mL of methanol). After 30min. in the dark at room temperature, the absorbance was measured against a blank at 515nm. The blank was a solution where 500µL of Trolox and 500µL of methanol reacted with 1mL of DPPH reagent to obtain the complete decoloration of that radical. For the calibration curve 0.5-3mM

of Trolox in 1mL of methanol was used and results were expressed as Trolox equivalent antioxidant capacity (TEAC) [7].

$$\text{Percent (\%) inhibition of DPPH activity} = \frac{A - B}{A} \times 100$$

Where A is the optical density of the blank and B is the optical density of the sample.

2.4 Acute Toxicity Experiment

Albino mice were divided into control and test groups (6 animals each). Control group received the vehicle (3% Tween 80) while the test groups got graded doses (1000-4000mg/kg) of *C. coronarium* ethanol extract orally and were observed for mortality till 48h.The dose selection for the ethanol extract of *C. coronarium*was based on the acute toxicity study, which did not show any adverse effect following oral administration of doses up to 4000mg/kg. Accordingly, experimental oral doses of 200 and 400mg/kg that equal to one-twentieth and one-tenth of the maximum possible dose of the extract that did not cause mortalities in mice.

2.5 Sub-chronic Toxicity

Eighteen male Wistar albino rats were randomly divided into 3 groups of 6 animals. The 1st group was kept as control (5mL/kg of 3% Tween 80), while 2nd and 3rd groups were administered the ethanol extract of *C. coronarium*in doses of 200 and 400mg/kg, respectively. All medications were administered orally with the aid of an orogastric cannula for 35 consecutive days. Rats were maintained under identical conditions with food and water *ad libitum* for the entire period with close observation. At the end of the experimental period, blood samples (2mL) were drawn by puncturing retro-orbital venous sinus of each rat (under ether anesthesia) and centrifuged at 10000 rpm for 5min. Sera were separated to be used for the biochemical estimations.

2.6 Experimental Induction of Hepatic Damage

$CCl_4$was dissolved in corn oil in the ratio 1:1 v/v. Liver damage was induced in rats following subcutaneous (SC) injection of CCl_4 in the lower abdomen at a dose of 3mL/kg [9].

2.7 Hepatoprotective Activity

Twenty-four male Wistar albino rats were randomly divided into four groups of six animals, each. Rats of the 1st (normal control) and 2nd (CCl_4-intoxicated control) groups received the vehicle in a dose of 5mL/kg. The 3th and4^{th} groups were treated with the ethanol extract of *C. coronarium* in doses of 200 and 400mg/kg, respectively. All medications were administered orally by gastric intubation for 7 consecutive days. Two h after the last dose, normal control rats were given a single dose of corn oil (3mL/kg, SC), while animals of the 2nd-4th groups received a single dose of CCl_4 (3mL/kg, SC). After 24h of corn oil and CCl_4 injections, blood samples from each rat (2mL) was withdrawn by puncturing their retro-orbital plexus of veins, collected in previously labeled centrifuging tubes, and allowed to clot for 30min at room temperature. Serum was separated by centrifugation at 10000 rpm for 5min.

2.8 Measurement of Liver and Kidney Function Markers

Liver functions were evaluated by measuring the serum activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) [10]. The serum concentrations of TB [11], TP [12] and Alb [13] were estimated. Serum levels of urea [14] and creatinine [15] were determined colorimetrically as measures of kidney functions.

2.9 Effect on Male Fertility

A total number of 18 male Wistar rats were divided into 3 groups of 6 animals each. The tested extract was given to the rats by gavage daily for 65 consecutive days. This administration period is necessary to determine the effect of the tested extracts on sperm production because the rats need a period of 65 days for the exact spermatogenic cycle [16]. Group I: Control rats received 0.5ml/day of the vehicle, i.e., Tween 80 in equivalent amount of normal saline. Groups II and III Rats were treated with *C. coronarium*at 200 and 400mg/kg, respectively.

2.10 Sample Collection

The animals were weighed and sacrificed under light ether anesthesia, 24h after last dose of the treatment. Blood samples were collected by cardiac puncture into centrifuge tubes and left to clot for 10min at room temperature. The tubes were centrifuged at 3000g for 5min and the sera were separated, stored frozen and used within 12h of preparation for the estimation of circulatory levels of hormones, namely, testosterone [17], prolactin [18], follicle-stimulating hormone (FSH) and luteinizing hormone (LH) [19].

2.11 Body and Relative Organ Weight Measurements

Initial and final body weights of the animals were recorded. The animals were dissected and testes, epididymis, seminal vesicle and ventral prostate were excised, cleared of adhering fat and connective tissue. Testes, seminal vesicle and ventral prostate were weighed to the nearest milligram on a digital electric balance. Organ weights were reported as relative weights (organ weight/body weight×100).

2.12 Sperm Characteristic Analysis

Right caudal epididymis was finely minced by anatomical scissors in 1mL of isotonic saline in a Petri dish. It was completely squashed by a tweezers for 2min, and then allowed to incubate at room temperature for 4h to provide the migration of all spermatozoa from epididymal tissue to fluid. The epididymal sperm concentration was determined with a hemocytometer using a modified method [20].

The percentage of forward progressive sperm motility was evaluated using a light microscope with heated stage [21]. The left cauda epididymis from each animal was incised and a very small droplet of epididymal fluid obtained with a pipette was dropped on the slide. Several drops of Tris buffer solution [0.3M Tris (hydroxymethyl) aminomethane, 0.027M glucose, 0.1M citric acid] were added to the epididymal fluid and mixed by a cover-slip. The percentage of forward progressive sperm motility was evaluated visually at 400×magnification. Motility estimates were performed from three different fields in each sample. The means of the three successive estimations were used as the final motility score.

A sperm viability test was done by the method described by World Health Organization [22] and spermabnormality [23].

2.13 Statistical Analysis

The values were expressed as mean± standard error of six observations in each group. All groups were subjected to one-way analysis of variance (ANOVA), which was followed by Bonferoni's test to determine the intergroup variability by using SPSS ver. 14.0. We took a P-value of <0.05 as our desired level of significance.

3. RESULTS AND DISCUSSION

3.1 Antioxidant Activity

Antioxidant activity was measured by the DPPH assay, by using this method, we can do many samples in a short period and it is sensitive to determine active components at low concentrations [24]. The scavenging activity of crude extract and different fractions (diethyl ether, chloroform, ethyl acetate and n-butanol extracts) were determined as shown in Table 1. The highest activity was observed in the ethyl acetate extract (49.45%) followed by the chloroform and total extracts (36.4 % and 34.85 % respectively). The butanol and diethyl ether extracts showed the lowest antioxidant activity (24.50% and 14.50% respectively). Rutin and apigenin showed antioxidant activity which appeared in inhibition of oxidation of beta-carotene and lipid peroxidation by the ammonium thiocyanate method. Methoxylated flavonoids exhibited a lesser antioxidant activity [25]. From previous studies *C. coronarium* was found to contain rutin, luteolin, luteolin-7-O-glucoside [6]. The antioxidant activity of *C. coronarium* may be due to the high flavonoid contents, in which rutin showed strong DPPH radical scavenging activity, at the concentration of 0.05 mg/ml, rutin also had inhibition of lipid peroxidation [26].

3.2 Acute Toxicity Experiment

All mice treated with different doses (1000-4000mg/kg) of *C. coronarium* extract survived during the 48h of observation. The animals did not show visible signs of acute toxicity.

3.3 Sub-chronic Toxicity Experiment

No significant changes were detected in the biochemical parameters of rats after 35 days of treatment with *C. coronarium* extract. Oral administration of the tested extract in doses of 200 and 400mg/kg to rats for 35 days did not show any significant effect on the levels of ALT, AST, TB, TP, Alb Table 2, also no significant changes were observed in urea and creatinine (data not shown) as compared to control animals.

3.4 Hepatoprotective Activity

SC injection of CCl_4 to rats showed significant elevation of liver marker enzymes (ALT, AST) in their serum after 24h of intoxication. The level of TB in the serum of CCl_4-intoxicated control was also significantly increased when compared to the normal control group. Administration of *C. coronarium* (400mg/kg) once daily for 7 days prior to CCl_4, exhibited a significant hepatoprotective activity, resulting in reduction in the elevated serum activities of liver marker enzymes Table 3.and level of TB when compared to CCl_4-intoxicated rats. This

hepatoprotective activity may be due to the anti-oxidant activity and flavonoid contents of *C. coronarium* [6], in which rutin was able to prevent the CCl_4-induced rise in serum enzymes, rutin also prevented the CC4-induced prolongation in pentobarbital sleeping time confirming its hepatoprotective activity [27].

3.5 Effect on Body and Relative Reproductive Organs Weights

The body weight of animals treated with *C. coronarium* in doses of 200 and 400mg/kg for 65 days did not change, indicating that the general metabolic conditions of the animals were within the normal range. Administration of the ethanol extracts of *C. coronarium* (400 to the rats for 65 days caused a significant increase in the relative weights of the testes, seminal vesicles and ventral prostate compared with the controls Table 4. The weights of the accessory sex organs require continuous androgenic stimulation for their normal growth and functions. Therefore, the increased weight of the sex organs could be attributed to the increased levels of serum LH and testosterone by *C. coronarium*.

3.6 Serum Hormone Levels

FSH, LH and testosterone are prime regulators of germ cell development. The quantitative production of spermatozoa generally requires the presence of FSH, LH and testosterone. FSH acts directly on the seminiferous tubules, whereas LH stimulates spermatogenesis indirectly via testosterone [28]. The effect of *C. coronarium* on serum hormone profile in male rats as shown in Table 5. The means of serum testosterone and LH levels of rats treated with *C. coronarium* (400mg/kg) for 65 days significantly increased compared with the controls. In fact, LH binds to Leydig cells and increases cAMP, which increases protein secretion and the side-chain cleavage of cholesterol, as well as other likely steps, to increase steroidogenesis and the production of testosterone and other androgens. In addition, the deficiency of LH and FSH prevents the gonads from either producing sperms or sufficient quality of testosterone [29].

The serum levels of prolactin and FSH did not reveal any significant change in all treated groups when compared with their control counterparts.

3.7 Epididymal Sperm Characteristics

The effects of 200 and 400mg/kg of *C. coronarium* extracts on sperm counts, motility, viability and abnormalities are shown in Table 6.

3.8 Epididymal Sperm Count

In Table 6, daily administration of *C. coronarium* (400mg/kg) extract to rats for 65 days significantly increased the means of epididymal sperm counts ($73.76\pm2.15\times10^6$ sperm/mL, compared with its control group ($65.85\pm2.68\times10^6$ sperm/mL). Testosterone in humans or and rostenedione in animals are synthesized in the Leydig cells under the influence of LH [30]. Thus, increased testosterone level is responsible for the increased sperm counts noted in *C. coronarium* (400mg/kg) treated group when compared with the control.

Table 1. Antioxidant activity of *C. coronarium* extract and their four fractions

Different extract	Inhibition %	TEAC
Total extract	34.85±2.15	0.92±0.06
Diethyl extract	14.50±1.2	0.54±0. 02
Chloroform extract	36.30 ±1.4	0.98±0.03
Ethyl acetate extract	49.45±1.45	1.65±0.4
Butanol extract	24. 50±2.4	1.29±0.02

TEAC: Trolox equivalent antioxidant capacity

Table 2. Sub-chronic effect of oral administration of ethanolic extract of *C. coronarium* for 35 days on the serum activity of ALT and AST and serum levels of total bilirubin, total protein and albumin in rats, (n=6)

Groups	ALT (UL^{-1})	AST (UL^{-1})	T. bilirubin (mg dL^{-1})	T. protein (g dL^{-1})	Albumin (g dL^{-1})
Control	68.63±3.15	141.12±5.83	1.39±0.06	8.1±0.24	3.4±0.11
C. coronarium (200mg/kg)	66.54±2.60	142.44±3.20	1.40±0.07	7.9±0.31	3.4±0.10
C. coronarium (400mg/kg)	63.78±2.79	143.25±4.21	1.46±0.08	7.8±0.36	3.6±0.12

AST: aspartate aminotransferase and ALT: alanine aminotransferase

Table 3. Effect of the ethanolic extract of *C. coronarium* on the serum activity of ALT and AST and serum levels of total bilirubin, total protein and albumin in rats with CCl$_4$ induced–hepatotoxicity

Groups	ALT (UL^{-1})	AST (UL^{-1})	T. bilirubin (mg dL^{-1})	T. protein (g dL^{-1})	Albumin (g dL^{-1})
Normal Control	68.0±2.11*	140.5±4.18*	1.24±0.06*	8.47±0.39*	3.65±0.18*
CCl$_4$-intoxicated Control	357.4±11.5	462.5±12.7	3.7±0.18	5.5±0.27	2.4±0.15
C. coronarium (200mg/kg)	302.5±7.5	411.5±10.3	3.4±0.16	5.8±0.22	2.6±0.11
C. coronarium (400mg/kg)	238.8±8.9*	345.9±11.4*	3.0±0.16*	6.4±0.32*	2.9±0.12*

The results are expressed as mean±S.E.M., n=6 rats/group., indicate significance compared to CCl$_4$ group (p<0.05)*

Table 4. Effect of oral administration of ethanolic extract of *C. coronarium* for 65 days on weights of sexual organs of male rats, (n=6)

Groups	Initial b.wt (g)	Final b.wt (g)	Weight of reproductive organs (g/100g b.wt)		
			Testes (Pair)	Seminal vesicles	Ventral prostate
Control	216.8±7.34	234.6±7.48	1.57±0.13	0.60±0.03	0.44±0.02
C. coronarium (200mg/kg)	224.5±6.6	246.5±7.1	1.68 ±0.11	0.64±0.02	0.47±0.02
C. coronarium (400mg/kg)	232.4±7.11	250.8±7.52	2.16±0.15*	0.73±0.03*	0.53±0.03*

*, indicate significance at (p<0.05)

Table 5. Effect of oral administration of ethanolic extract of *C. coronarium* for 65 days on plasma levels of reproductive hormones of male rats, (n=6)

Groups	Testosterone (ng mL^{-1})	Prolactin (ng mL^{-1})	FSH (mIU mL^{-1})	LH (mIU mL^{-1})
Control	4.45±0.23	0.72±0.04	7.32±0.22	0.62±0.04
C. coronarium (200mg/kg)	5.62±0.31	0.69±0.02	7.20±0.30	0.71±0.02
C. coronarium (400mg/kg)	7.52±0.37*	0.65±0.03	7.00±0.36	0.83±0.06*

*, indicate significance at (p< 0.05).

Table 6. Effect of oral administration of ethanolic extract of *C. coronarium* for 65 days on semen characteristics of male rats, (n=6)

Groups	Sperm count (X 10^6/mL)	Sperm motility (%)	Unstained (live) sperms (%)	Total sperm abnormality (%)
Control	65.85±2.68	89.90±4.72	93.43±4.35	3.77±0.15
C. coronarium (200mg/kg)	68.45±2.1	91.44±3.2	93.44±4.45	3.72±014
C. coronarium (400mg/kg)	73.76±2.15*	94.68±4.86	93.52±4.73	3.23±0.14*

*, indicate significance at (p<0.05)

3.9 Sperm Motility

Oral administration of the total ethanol extract of *C. coronarium* (400mg/kg) for 65 days, slightly increased sperm progressive motility compared with the control group. Seminal vesicle secretes fructose, phosphorylcholine, ergothioneine and prostaglandins, these chemical components of seminal fluid are responsible for enhancing motility of sperm; hence, its increased secretion by the organ will lead to increased motility [31].

3.10 Sperm Viability and Abnormalities

The sperm viability did not reveal any significant change in all treated group when compared with their control group. The percentages of these abnormalities were significantly reduced Table 6 in groups medicated with *C. coronarium* (400mg/kg) extract when compared to the control group. This fertility promoting activity of *C. coronarium* may be related to its high anti-oxidant and flavonoid content. From previous studiesanimals treated with 5mg/kg and 10mg/kg of rutin and naringin (flavonoids) have shown significant and dose dependent reduction in malanoldehyde levels and increase in levels of antioxidant enzymes, super oxide dismutase and Catalase when compared to control group animals. Sperm count, motility, viability were also protected and normalized with rutin and naringin [32].

4. CONCLUSION

The results suggest that *C. coronarium* has a significant hepatoprotective activity and promote fertility at conc. of 400mg/kg, also further studies needed to isolate the pure active constituents which responsible for these activity.

ETHICAL APPROVAL

ACKNOWLEDGEMENT

COMPETING INTERESTS

REFERENCES

1. Basta A, Pavlovi´ M, Couladis M, Tzakou O. Essential oil composition of the flowerheads of *Chrysanthemum coronarium* L. from Greece. Flavour Frag. J. 2007;22:197–200.
2. Chuda Y, Ono H, Ohnishi-Kameyama M, Nagata T, Tsushida T. Structural identification of two antioxidant quinic acid derivatives from garland (*Chrysanthemum coronarium* L.). J. Agric. Food Chem. 1996;44:2037–2039.
3. Alvarez-Castellanos PP, Bishop CD, Pascual-Villalobos MJ. Antifungal activity of the essential oil of flowerheads of garland chrysanthemum (*Chrysanthemum coronarium*) against agricultural pathogens. Phytochemistry. 2001;57:99–102.
4. Lii CK, Lei YP, Yao HT, Hsieh YS, Tsai CW, Liu KL, Chen HW. *Chrysanthemum morifolium* Ramat. Reduces the oxidized LDL-induced expression of intercellular adhesion molecule-1 and E-selectin in human umbilical vein endothelial cells. J. Ethnopharmacol. 2010;128:213–220.
5. Lai JP, Lima YH, Sua J, Shen HM, Ong CN. Identification and characterization of major flavonoids and caffeoylquinic acids in three Compositae plants by LC/DAD-APCI/MS. J. Chromatogr. 2007;848:215–225.
6. Hosnia K, Imed H, Houcine S, Hervé C. Secondary metabolites from *Chrysanthemum coronarium* (Garland) flower heads: Chemical composition and biological activities. Industrial Crops and Products. 2013;44:263–271.
7. Liu M, Li XQ, Weber C, Lee CY, Brown J, Liu RH. Antioxidant and antiproliferative activities of raspberries. J. Agric. Food Chem. 2012;50:2926-2930.
8. Ghosh MN. Fundamentals of Experimental Pharmacology, 2nd Edn., Scientific book agency, Kolkatta. 1994;153-158.
9. Theophile D, Laure NE, Benoit NT, Anatole AGB, Emmanual AA, Pual TV, Pierre K. Antinociceptive and Anti-inflammatory effects of the ethyl acetate stem bark extract of Brideliascleroneura. Inflamm-pharmacol. 2006;14:42-47.
10. Reitman S, Frankel S. Colorimetric methods for aspartate and alanine monotransferases. Am. J. Clin. Path. 1957;28:55-60.

11. Walter M, Gerarade H. Ultramicro method for the determination of conjugated and total bilirubin in serum or plasma. Microche. J. 1970;15:231.

12. Henary RJ, Cannon DC, Winkleman JW. Clinical Chemistry Principles and Techniques 2nd Ed. Harper and Roe, New York; 1974.

13. Doumas BT, Watson WA, Biggs HG. Albumin standards and the measurement of serum albumin with bromocresol green. Clin. Chim. Acta. 1971;31:87-96.

14. Wills MR, Savory J. Biochemistry of renal failure. Ann. Clin. Labo. Sci. 1981;11:292-299.

15. Kroll MH, Roach NA, Poe B, Elin RJ. Mechanism of interference with Jaffé reaction for creatinine. Clini. Chem. 1987;33:1129-1132.

16. Amann RP. Use of animal models for detecting specific alterations in reproduction. Fund Appl. Toxicol. 1982;2:13-26.

17. Chen A, Bookstein JJ, Meldrum DR. Diagnosis of a testosterone-screening adrenal adenoma by selective venous catheterization. Fertil. Steril. 1991;55:1202-1203.

18. Tietz NW. Clinical Guide to Laboratory Tests. 3rd Edition, W.B. saunders, Co. 1991;512-513.

19. Uotila M, Ruoslahti E, Engvall E. Relative conversion of arachidonic acid through lipoxygenase and cyclooxygenase pathways by homogenates of diseased periodontal tissues. J. Immunol. Methods.1981;42:11-15.

20. Sönmez M, Yuce A, Turk, G. The protective effects of melatonin and vitamin E on antioxidant enzyme activities and epididymal sperm characteristics of homocysteine treated male rats. Reprod. Toxicol. 2007;23:226-231.

21. Sönmez M, Turk G, Yuce A. The effect of ascorbic acid supplementation on sperm quality, lipid peroxidation and testosterone levels of male Wistar rats. Theriogenology. 2005;63:2063–2072.

22. WHO. Laboratory Manual for the examination of the human semen and Sperm-cervical mucus interaction. New York: Cambridge University Press; 1999.

23. Türk G, Atessahin A, Sonmez M, Yuce A, Ceribasi AO. Lycopene protects against cyclosporine A-induced testicular toxicity in rats. Theriogenology. 2007;67:778–785.

24. Sa´nchez-Moreno C. Methods used to evaluate the free radical scavenging activity in foods and biological systems. Food Sci. Technol. Int. 2002;8:121-137.

25. Sharififar F, Dehghn-Nudeh G and Mirta Jaldini M. MaJor flavonoids with antioxidant activity from *Teucrium polium* L. Food Chem. 2009;112:885-888

26. Yang J, Guoa J, Yuan J. In vitro antioxidant properties of rutin. LWT. 2008;41:1060-1066.

27. Janbaz KH, Sheikh AS, Anwar HG. Protective effect of rutin on paracetamol-and CC14-induced hepatotoxicity in rodents. Fitoterapia. 2002;73:557-563.

28. Anderson RA, Wallace EM, Groome NP, Bellis AJ, Wu FCW. Physiological relationships between inhibin B, follicle stimulating hormone secretion and spermatogenesis in normal men and response to gonadotrophin suppression by exogenous testosterone. Hum. Reprod. 1997;12:746-747.

29. Nieschlag E. Classification of andrological disorders. In: Nieschlag E, Behre HM editors. *Andrology;* Male Reproductive Health and Dysfunction. Berlin, Springer. 1997;81-86.

30. Vasudevan DM, Sreekumari S. Textbook of Biochemistry for Medical Students. 4th Edition, Jaypee Brothers Medical Publishers (P) LTD, New Delhi, India. 2005;371-377.

31. Ganong W. Review of medical Physiology. 20th ed. McGraw-Hill; 1999.

32. Akondi RB, Annapurna A, Siva RC. Protective Effects of Rutin and Naringin on Gentamycin Induced Testicular Oxidative Stress. Eur. J. Gen. Med. 2011;8:57-64.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=506&id=32&aid=4417