HYPOCHOLESTEROLEMIC EFFECTS OF *ADIANUM CAPILLUS VENERIS* L. AQUEOUS EXTRACT IN HIGH CHOLESTEROL DIET-FED RATS AND HPLC-MS DETERMINATION OF ITS POLYPHENOLICS

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Hypercholesterolemia is a predominant risk factor for atherosclerosis and associated with coronary and cerebrovascular diseases. The global incidence of hypercholesterolemia is rapidly increasing. *Adiantum capillus veneris* L. (Adiantaceae) has been reported as herbal medicine for diverse ailments in Jordan. However, its alleged benefits and action mechanisms remain elusive. Thus the present study aimed to identify the phenolic constituents of *A. capillus veneris* by LC-MS and to evaluate the chronic effect of its water extract on 10-weeks high cholesterol diet (HCD)-fed rats serum lipid profile and atherogenic index. Antioxidant potential is also assessed. Rats were divided into four groups of 6 rats each. Group 1 was kept on standard diet; the remaining three groups were high-cholesterol fed for ten weeks. On week 7 onwards; Group 3 was atorvastatin 10 mg/Kg b.wt- treated, while group 4 was supplemented daily with *A. capillus veneris* 500 mg/Kg b.wt. HPLC-MS analyses revealed the presence of ellagic acid (5.48 mg/g), rutin (4.77 mg/g), quercetin-3-O-glucoside (3.96 mg/g), ferulic acid (3.88 mg/g), gallic acid (3.44 mg/g), caffeic acid (1.55 mg/g), epicatechin (1.34 mg/g) and quercetine (0.43 mg/g). Crude extracts of the aerial parts exhibited potent antioxidant activity by ORAC, ABTS and DPPH assays (2184.95±109.3, 762.50±38.1, and 337.07±16.9 Trolox equivalent µmol/mg, respectively). Exceeding atorvastatin 10 mg/Kg b.wt effectiveness (p<0.05, n=6 rats/group), 10-week administration of *A. capillus veneris* extract in HCD-fed rats decreased highly significantly the total cholesterol (TC), LDL and VLDL serum levels. VLDL serum levels in both intervention groups were substantially (p<0.001) and comparably decreased. Neither treatment could affect HDL serum levels. Besides, atherogenic index parameter of TC/HDL was normalized in *A. capillus veneris*-treated rats. *A. capillus veneris* can be considered as potential candidate for management of hypercholesterolemia and its atherosclerotic complications.

INTRODUCTION

Hypercholesterolemia is a major cardiovascular risk factor that increases the incidence of atherosclerotic diseases.1 The role of statins – as hydroxymethylglutarylCoA (HMG-CoA) inhibitors – is well characterized for the reduction of cholesterol levels.2 Recent multiple studies on medicinal plants and their phyto-principles as well as food supplements effectiveness as safe and effective cholesterol-lowering agents were comprehensively reported. They have become progressively more attractive alternatives for alleviation of hypercholesterolemia.3-14
Adiantum capillus-veneris L. (Adiantaceae) known in English as “Southern maidenhair fern”, “black maidenhair fern”, “common maidenhair” and “venus hair fern” is considered among the medicinal plants identified by the locals for traditional medication and treatment of various diseases in Jordan. Multiple reports were investigating its diverse biological efficacies as well as its rich phytochemistry. Antimicrobial flavonoids were detected in A. capillus-veneris. Antioxidative properties were ascribed as well to A. capillus-veneris due to its phenolic and flavonoid contents. Besides its triterpenoidal compounds, marked anti-inflammatory and significant hypoglycemic activities were recognized for A. capillus-veneris. Additionally substantial analgesic effects and antiurolithic activities were attributed to A. capillus-veneris. Angiogenic effects and protective effects against oxygen free radicals and wound healing potential were reported recently for different A. capillus-veneris extracts. Although this traditionally widely used fern biologically and phytochemically screened in many Middle Eastern countries, no studies were reported from Jordan. Hence, the objective of this study was to investigate the chronic cholesterol-lowering role of A. capillus veneris aqueous extract in atherogenic diet fed rats and identify the phenolic constituents using LC-MS.

EXPERIMENTAL

Plant material and extract preparation

Dried aerial parts of A. capillus-veneris were provided by one of the authors (Prof. K. Abdul-Razzak) and identified macroscopically using descriptive reference and in comparison with the herbarium specimens of the Herbarium of the Department of Biological Sciences, Faculty of Science, The University of Jordan. Voucher specimens of the plant material were deposited at the Department of Pharmaceutical Sciences, The University of Jordan (Reference No. 127). Aqueous (AEs) extracts were prepared as described earlier.

In brief AEs were prepared by refluxing (without boiling) each 10 g of the dried coarsely powdered plant material with 100 mL tap water for 15 min. The overnight kept extracts were filtered twice through filter paper and the volume of the filtered solution was increased to 100 mL with tap water to obtain 10% (equivalent to 100 mg/1 mL) crude aqueous solutions. Sonication of stock crude extract or testing concentrations was performed before implementation of investigations.

Reagents and equipments

The stock solutions (1 mg/mL) of the reference substances caffeic acid, ferulic acid, ellagic acid, gallic acid, epicatechin, quercetin, rutin, quercetin 3-β-D-glucoside, luteolin, kaempferol,isorhamnetin and resveratrol were prepared in methanol and were stored at -40 °C between the experiments. All the other reagents, (acetonitrile, formic acid, Merck, Germany) were analytical pure or of chromatographic grade and they were used after filtration. The ultra-pure water was obtained using a system for purification of water, Elix 3 (Millipore, USA).

The chromatographic measurements were performed using a complete HPLC SHIMADZU system, using a Nucleosil 100C18 column with size particle 3.5 μm, KROMASIL, 100x2.1 mm. The system was coupled to a MS detector, LCMS-2010 detector (liquid chromatograph mass spectrometer), equipped with an ESI interface. The mobile phase was sonicated in order to eliminate the dissolved air and then subjected to filtration using a PTFE 0.2 μm membrane. The samples were filtrated before injection using Syringe Driven Filter Unit 0.2 μm (Macherey-Nagel, Germany).

HPLC analysis

The column was equilibrated for 1 hour before injections were started. The mobile phase was a gradient prepared from formic acid in water (pH=3, solvent A) and formic acid in acetonitrile (pH=3, solvent B): 0.01-20.00 min 5-30% solvent B; 20.00-40 min 30% solvent B; 40.01-50.00 min 30-50% solvent B; 50.01-52.00 50-5% solvent B; 52.01-70.00 5% solvent B. The flow rate was: 0.01-5.00 min 0.1 mL/min; 5.01-15 min 0.2 mL/min; 15.01-35 min 0.1 mL/min; 35.01-60 min 0.2 mL/min; 60-70 min 0.1 mL/min. The analyses were performed at 20 °C for the period of 70 minutes. Then the column was washed over a period of 15 minutes with mobile phase using the flow rate 0.1 mL/min. After completion of series of analyses, the HPLC system was cleaned with water and methanol for 1 hour.

ESI source and negative ionisation mode was used. Nitrogen was used as the nebulising and drying gas. The SCAN (m/z 50-800) mode was used for general identification of compounds and the SIM mode (m/z ranging from 50-800) was used when a search for some particular ions and the quantification of the corresponding compounds should be done.

Antioxidant efficacy and free radical scavenger assessment

The free radical scavenging activity was evaluated using 2,2’-diphenyl-1-pyridyl hydrazyl assay (DPPH Assay) and 2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)-radical scavenging activity assay and expressed as Trolox Equivalent Antioxidant Capacity (TEAC). The antioxidant efficacy was determined using Oxygen Radical Antioxidant Capacity (ORAC) thermoeVolution UV-Vis spectrometer (USA) was used for DPPH and TEAC assays, measurements being performed at specific wavelengths corresonding to maximum absorbency for DPPH, 515 nm, respectively ABTS cation radical, 732 nm. All results were expressed as Trolox equivalent, in micromols per mg. The ORAC assay was carried out using an OCEAn Optics fluorimeter with optical fiber and having as fluorescent probe fluorescein. Even in this case the antioxidant efficacy was expressed as Trolox equivalent, in micromols per mg.

Experimental animal groups

The study was conducted at the Experimental Animal Laboratory of the Department of Biological Science, Faculty of Science; The University of Jordan. All animals were
housed, fed and treated in accordance with the University of Jordan ethical guidelines for animal protection and experimental approval. It also fulfills the accepted international requirements in this field. Throughout the experimental period, animals were kept in single cages. Locally inbred male Wistar rats of 212.5 ± 1.9 g average body weight (b.wt) were used in the experiments. Rats were provided with normal diet chow (called basal diet hereafter) and water ad libitum for the duration of the experiment except during the 12-13 hrs fasting period preceding cholesterol administration and blood collection. Rats were divided into 4 groups (n=6) as follows: 

**Group 1:** The negative control group was given only the control diet ad libitum for 10 weeks.

**Group 2:** The hypercholesterolemic control group was given the control diet ad libitum and high cholesterol diet (HCD) once daily for 10 weeks.

**Group 3:** The treatment group was given the control diet ad libitum and HCD once daily for 10 weeks, while *A. capillus-veneris* 500 mg/Kg b.wt was fed in the last four weeks once daily for the last 4 weeks (week 7-10).

**Group 4:** The positive control group was given the control diet ad libitum and HCD once daily for 10 weeks. To this group atorvastatin (10 mg/kg) was administered once daily for the 4 weeks (weeks 7-10). Atorvastatin was administered to rats daily at 5:00-6:00 pm.

**Determination of median lethal dose LD50**

Locally inbred mice (*Mus musculus*) of both sexes with average body weight (b.wt) of 24.25 ± 1.59 g were used for determination of LD50 of water extract. Mice of both sexes were divided into 2 groups each of 4 mice (2 males and 2 females).

Group 1 served as a control (untreated, given only distilled water), group 2 was given the aqueous extract. Mice were fasted overnight before treatment and they were observed for lethality over a period of 24 hrs and for the following 7 days.

For the determination of LD50, a single dose of 1 ml/mouse was administered orally. The highest dose administered was 8g/Kg b.wt (no infusion could be prepared in higher concentration with the plant material), no fatalities were encountered. Accordingly, 500 mg/Kg (equivalent to 6%) of the highest prepared dose was selected; lethality was observed neither after 24hrs nor after one week. Moreover, all animals exhibited normal behaviour.

At the end of day 7, animals were sacrificed; macroscopically gross anatomy and blood biochemistry were performed. Blood was collected in heparinised capillary tubes from all mice of groups 1 and 2 for the determination of red blood cells (RBC), white blood cells (WBC) and hemoglobin (Hb). RBC and WBC were counted manually using Newbauer counting chamber, while Hb was determined according to Sahli method. In brief, HCL, 0.1 N, is placed in the graduated tube of the Sahli outfit, 20 µl of blood is added to yield acid hematin. HCL was added drop wise until the colour of the tube corresponds exactly to the colour of the standard. Readings from the graduated tube are multiplied by 17.3, the Sahli standard.

**Crude extract administration**

*A. capillus-veneris* aqueous extract (AE) was administered to animals by gavage at 6:30 am daily. AE dose was selected based on the preliminary LD50 screening. Blood samples were collected weekly from the retro orbital plexus of rats using a 10 µL glass capillary. A blood sample was also collected just before the start of the experiment to measure the control values for each parameter for each animal group. All blood samples were collected after 12-13 h of fasting. Serum was collected from blood by centrifugation for 10 min at 3500 rpm and then stored at -80°C until analysis. Analysis was conducted in duplicate and was completed within 1-3 days of collection. As study was on HFD rats, therefore quantitative determination of lipid profiles (cholesterol, HDL, LDL and VLDL) were achieved using enzymatically commercial available kits (Lab kits, Barcelona, Spain).

**Atherogenic index**

Due to the fact that hypercholesterolemia is positively correlated with coronary heart diseases (CHD), it was noteworthy to check TC/HDL and HDL/LDL ratios as indicators of CHD predictors.

**Statistical analysis**

The values are presented as mean ± S.E.M. (Standard Error of the Mean) of 3-4 independent experiments. Statistical differences between control and different treatment groups and A.U.Cs (incremental Area Under 24h-glucose Curve) were determined using Graphpad Prism one way analysis of variance (ANOVA) followed by Dunnett post test whenever appropriate (version 3.02 for windows; GraphPad Software, San Diego, CA, USA). Values were considered significantly different if \( P<0.05 \) and highly significantly different if \( P<0.01 \) and \( P<0.001 \).

**RESULTS AND DISCUSSION**

**HPLC-MS analysis and antioxidant activity**

HPLC-MS analysis of the ethanol extract of *A. capillus-veneris* resulted in the identification of several flavonoids, catechins and cinnamic acid derivatives. The concentrations of the identified substances, given in mg/g ethanol extract indicated that ellagic acid (5.48 mg/g) is the major polyphenolic compound, followed by rutin (4.77 mg/g), queretin-3-O-glucoside (3.96 mg/g), ferulic acid (3.88 mg/g), gallic acid (3.44 mg/g), caffeic acid (1.55 mg/g), epicatechine (1.34 mg/g) and quercetine (0.43 mg/g), the corresponding chromatogram is presented as Fig. 1. Accordingly, the obtained results were leading to an expected high free-radical scavenging activity, obtained values given in Table 1.

**Median lethal dose (LD50)**

Blood parameters were within normal range compared to the untreated animals as shown in Table 2. No organs abnormalities were observed. Accordingly, it was decided to continue the chronic study with the dose of 500 mg/Kg. b.wt...
based on its safety profile. This selected dose corresponds to 35 g of the plant material/ 70 Kg b.wt (average human b.wt). This represents approximately a handful of plant material used in the traditional medicine for the preparation of herbal remedies.

![HPLC-MS chromatogram of A. capillus veneris extract. Inset mass spectra of the main identified compounds. (1) gallic acid; (2) caffeic acid; (3) rutin; (4) quercetin-3-O-glucoside; (5) ferulic acid; (6) ellagic acid; (7) quercetin.]

**Table 1**

<table>
<thead>
<tr>
<th></th>
<th>ORAC assay expressed as Trolox equivalent µmol/mg</th>
<th>ABTS assay expressed as Trolox equivalent µmol/mg</th>
<th>DPPH assay expressed as Trolox equivalent µmol/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adiantum capillus veneris (aerial parts)</td>
<td>2184.95±109.3</td>
<td>762.50±38.1</td>
<td>337.07±16.9</td>
</tr>
</tbody>
</table>

All the results are expressed as trolox equivalent in micromols per mg are mean ± SEM (n = 3).

**Table 2**

Results of RBC, WBC and haemoglobin of different groups of mice used in LD50 determination (n= 4 of both sexes/ group)

<table>
<thead>
<tr>
<th>Group Name</th>
<th>RBC /mm³</th>
<th>WBC / mm³</th>
<th>Hb (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>(4.70 ± 0.20) x 10⁶</td>
<td>(10.65 ± 0.51) x 10³</td>
<td>11.70± 0.10</td>
</tr>
<tr>
<td>(20%) Water extract</td>
<td>(6.60 ± 0.34) x 10⁶</td>
<td>(7.90 ± 0.50) x 10³</td>
<td>13.75± 0.24</td>
</tr>
</tbody>
</table>

Data are expressed as Means± SEM.
Chronic effects of *A. capillus veneris* on serum lipid profile

**Chronic effects of *A. capillus veneris* on serum total cholesterol (TC)**

Fig. 2 demonstrates TC AUC 10 weeks in HCD fed-animals is significantly greater than control rats (1883.8 ± 73.5 vs. 825.8±16.1, n=5-6 rats/group, p<0.001). Additionally, TC AUC 10 weeks in atorvastatin -HCD fed rats is highly significantly greater than control animals’ (1396.7± 63.2 vs. 825.8± 16.1, n=6 rats/group, p<0.001). Interestingly, TC AUC 10 weeks in atorvastatin-HCD fed rats is substantially less than HCD-fed rats (1396.7 ± 63.2 vs. 1883.8 ± 73.5, n=5-6 rats/group, p<0.001). TC AUC 10 weeks in *A. capillus veneris* 500 mg/Kg b.wt -HCD fed animals is highly substantially more than controls’ (1191.5 ± 55.3 vs. 825.8±16.1, n=6 rats/group, p<0.001). Nevertheless, TC AUC 10 weeks in *A. capillus veneris*-HCD fed rats is pronouncedly decreased vs. HCD-fed rats (1191.5 ± 55.3 vs. 1883.8 ± 73.5, n=5-6 rats/group, p<0.001). Most impressively, TC AUC 10 weeks in *A. capillus veneris*-HCD fed rats is pronouncedly decreased vs. hypocholesterolemic drug atorvastatin HCD-fed rats (1191.5 ± 55.3 vs. 1396.7 ± 63.2 , n=6 rats/group, p<0.05). Hyperoside as well as chlorogenic acid were strongly associated with HMG-CoA reductase inhibition via synergistic action with quercetine.35-36 Further inhibitory potential on hepatic cholesterol synthesis was related to artichoke content of chlorogenic acid.37 Additionally, as Hawthorn combination with simvastatin exhibited a substantial lipid lowering effectiveness in hyperlipidemic albino rats.38 Unequivocally, Hawthorn fruit active principles, inhibiting synergistically HMG-CoA reductase and cholesterol absorption could manifest significant hypolipidemic benefits, further validating the hypocholesterolemic effect of *C. aronia* in high cholesterol diet fed rats.39-41 Effectively, the results indicate that the therapeutic cholesterol lowering efficacy of *A. capillus veneris* may be attributable to its multiple components acting additively or synergistically.

Fig. 2 – *In vivo* chronic effects of *A. capillus veneris* AE on the total cholesterol concentrations and the incremental AUC of 10 weeks treatment. ***P<0.001 compared to control group, ΔΔΔP<0.001 compared to HCD and *P<0.05 compared to atorvastatin 10 mg/Kg b.wt; as determined by ANOVA followed by Dunnett post test.
**Chronic effects of A. capillus veneris on serum LDL**

Fig. 3 illustrates that LDL AUC 10 weeks in HCD fed-animals is highly significantly greater than control rats' (1270.6 ± 104.2 vs. 222.9±11.1, n=5-6 rats/group, p<0.001). LDL AUC 10 weeks in atorvastatin-HCD fed rats is substantially less than HCD-fed rats (814.1 ± 78.1 vs. 1270.6±104.2, n=6 rats/group, p<0.001), though LDL AUC 10 weeks in atorvastatin-HCD fed rats is still markedly greater than control rats (814.1 ± 78.1 vs. 222.9±11.1, n=6 rats/group, p<0.001). In the same capacity, LDL AUC 10 weeks in *A. capillus veneris* 500 mg/Kg b.wt -HCD fed rats is highly significantly less than HCD-fed animals' (671.4 ± 39 vs. 1270.6±104.2, n=6 rats/group, p<0.001). Besides, LDL AUC 10 weeks in *A. capillus veneris* 500 mg/Kg b.wt -HCD fed animals is highly substantially greater than control group's (671.4 ± 39 vs. 222.9±11.1, n=6 rats/group, p<0.001). Therapeutic efficacies of hypo-cholesterolemic drug atorvastatin and *A. capillus veneris* were comparable (814.1±78.1 vs. 671.4±39.0, n=6 rats/intervention group, p>0.05).

**Chronic effects of A. capillus veneris on serum VLDL**

Fig. 4 illustrates that VLDL AUC 10 weeks in HCD fed-animals is significantly greater than control rats' (129.7 ± 6.7 vs. 107.8± 4.5, n=5-6 rats/group, p<0.05). VLDL AUC 10 weeks in atorvastatin-HCD fed rats is substantially less than HCD-fed rats (109.7 ± 3.6 vs. 129.7±6.7, n=5-6 rats/group, p<0.05), and, most essentially, normalized to control rats' (109.7 ± 3.6 vs. 107.8± 4.5, n=6 rats/group, p>0.05). Interestingly, VLDL AUC 10 weeks in *A. capillus veneris* 500 mg/Kg b.wt -HCD fed rats is highly significantly less than HCD-fed animals' (88.6 ± 3.6 vs. 129.7±6.7, n=5-6 rats/group, p<0.001). Nevertheless, VLDL AUC 10 weeks in *A. capillus veneris* 500 mg/Kg b.wt -HCD fed animals is substantially less than control group’s (88.6 ± 3.6 vs. 107.8± 4.5, n=6 rats/group, p<0.01). Incomparably, therapeutic efficacies of *A. capillus veneris* 500 mg/Kg b.wt -HCD fed animals is markedly greater than hypocholesterolemic drug atorvastatin's (88.6 ± 3.6 vs. 109.7 ± 3.6, n=6 rats/intervention group, p<0.05). This may be considered seriously into future herbal dosage titration, as safety parallel to effectiveness should be weighed equally not to the mutual exclusion of either.

**Chronic effects of A. capillus veneris on serum HDL**

Fig. 5 further reports the comparable ineffectiveness of both *A. capillus veneris* and atorvastatin on serum HDL in HCD-fed rats. None of the HDL AUC 10 weeks in HCD-fed animals or intervention groups were markedly changed as compared to control rats’ (454.8±32.4, 474.7±14.3 or 467.1±26.7 vs. 515.6±7.1, n=6 rats/ group, p>0.05).

**Chronic effects of A. capillus veneris on atherogenic index**

Importantly, amelioration of the different ratios such as HDL/LDL is considered as target for preventing/ reduction risk of CHD. Table 3 shows the ratios of TC/HDL and HDL/LDL at the end of 10 weeks. HCD group had significantly higher TC/HDL ratio (p<0.05) and lower HDL/LDL ratio (p<0.01) when compared to control group. As expected, TC/HDL ratio was normalized in both atorvastatin and *A. capillus veneris* rats. Therapeutic antiatherogenic activity can be attributed to be *A. capillus veneris* chronic administration. Interestingly, anti-atherogenic effect was correlated with chlorogenic acid-rich *Crataegus pinnatifida* in high fat diet fed rats. Furthermore, compared to standard diet rats, HDL/LDL ratio was markedly (p<0.01) decreased in HCD animals. Neither treatment groups could exhibit amelioration of HDL/LDL ratios, as both lacked HDL-restorative action (Fig. 5). These outcomes were closely comparable to those obtained with pine nuts chronic effects on hypocholesterolemia-related atherogenic indecies in high fat diet fed rats.

**CONCLUSIONS**

Succinctly *A. capillus veneris* phytochemicals may inhibit HMG-CoA reductase, in a similar action mode to statins, thus advocating phytotherapeutic strategy in atherosclerosis-related hypercholesterolemia.

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Fig. 3 – *In vivo* chronic effects of *A. capillus veneris* AE on the LDL concentrations and the incremental AUC of 10 weeks treatment. ***P<0.001 compared to control group, ∆∆∆P<0.001 compared to HCD, as determined by ANOVA followed by Dunnett post test.

Fig. 4 – *In vivo* chronic effects of *A. capillus veneris* AE on the VLDL concentrations and the incremental AUC of 10 weeks treatment. *P<0.05 and **P<0.01 compared to control group, ∆∆∆P<0.001 compared to HCD and ∆∇P<0.05 compared to atorvastatin 10 mg/Kg b.wt; as determined by ANOVA followed by Dunnett post test.
Fig. 5 – *In vivo* chronic effects of *A. capillus veneris* AE on the HDL concentrations and the incremental AUC of 10 weeks treatment.

**Table 3**

*In vivo* effects of *A. capillus veneris* extracts on TC/ HDL and HDL/LDL ratios

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HCD</th>
<th><em>Adiantum capillus veneris</em> 500 mg/Kg b.wt</th>
<th>Atorvastatin 10 mg/kg b.wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC/HDL</td>
<td>1.1± 0.1 Δ</td>
<td>1.4±0.1*</td>
<td>1.3±0.1 ∆∆▼</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>HDL/LDL</td>
<td>3.5±0.8 ∆∆▼</td>
<td>0.9±0.5**</td>
<td>1.1±0.5**</td>
<td>0.9±0.1*</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM (n = 6). *P<0.05, **P<0.01 compared to control group, ∆P<0.05, ∆∆P<0.01 compared to HCD group, and ▼ P<0.05 compared to atorvastatin 10 mg/Kg b.wt; as determined by ANOVA followed by Dunnett post test.

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