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Assessment of Usnic Acid (Lichen *Usnea Articulata* Extract) Safety on Lipid Profile, Adipocytes Morphology and Liver Functions in Adult Male Rats

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Abstract. Absorption and plasma level of usnic acid and safety of lichen *Usnea articulate* extract, usnic acid, were assessed in adult male Sprague-Dawley rats. Three groups (N = 10) were used: Group I received [1% carboxymethylcellulose (CMC)], Group II received extract/CMC (100 mg/kg), and Group III received extract/CMC (300 mg/kg) orally for 7 weeks. Usnic acid reached peak within 6 hr, remained in circulation for 72 hr after oral administration of 500 mg UA/kg. The extract results in significant increase in food intake at 2nd, 4th, 5th weeks in Group III versus controls. At 7th week, Group II showed an increase in albumin, total bilirubin, total cholesterol and high density lipoprotein cholesterol (HDL-C). In Group III glucose, magnesium, total bilirubin, alanine aminotransferase (ALT), total cholesterol, HDL-C, low density lipoprotein cholesterol (LDL-C), liver index were increased while aspartate aminotransferase (AST), lactate dehydrogenase (LDH) were decreased. Group II showed an increase in food efficiency, (LDH) but a decrease in glucose, magnesium, total bilirubin, (ALT), total cholesterol, LDL-C, HDL-C and liver index versus Group III. *Usnea articulate* extract exerted dose-dependent effects on body weight, liver functions, lipid profile and glucose with potential safety in low dose; affected adipocyte morphology and morphometry in high dose.

Keywords: Adipocyte morphology, Adipocyte morphometry**,** Liver functions, Perirenal fat index, *Usnea articulate*.

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Introduction

Obesity is an excessive accumulation of body fat, which poses a real threat to health. Its prevalence is increasing globally, particularly among children and adolescents, and nearly half a billion of the world's population (estimated to be around 6.5 billion) is considered to be overweight or obese[1]. Several anti-obesity agents (including drugs, nutritional and herbal dietary supplements) have been suggested for body weight control.

Usnea species have been used in homeopathic and traditional medicine in China, Pacific Islands, and New Zealand[2]. In Saudi Arabia, thirteen species of lichens from Asir mountains (Southwestern region bordering the Red Sea) were reported[3], while the flora of central, southern, and western regions were studied and about 67 lichen species belonging to 38 genera including *Usnea articulata* were recorded[4]. Twelve species belonging to eight genera were also identified[5]. Usnic acid (UA) is one of the most common and abundant secondary metabolites found in a variety of lichens. It exists as (+) and (-) enantiomers, both exhibiting unique biological properties. The (+)-usnic acid enantiomer exhibits anti-microbial[6], anti-inflammatory [7], and anti-viral activity[8] whereas (-)-usnic acid possess anti-fungal[9] and anti-mitotic properties[10]. The therapeutic antimicrobial activity of (+)-usnic acid against a large variety of Gram-positive bacteria, irrespective of their resistance phenotype, has been related to its membrane uncoupling property[11]. The anti-inflammatory, analgesic, and anti-pyretic effects of usnic acid have been linked to inhibition of prostaglandin synthesis as a result of uncoupling effects on oxidative phosphorylation[2].

The uncoupling property of UA provides the rationale for their use as fat burning agents and therefore, had been marketed as dietary supplements for weight loss[12]. However, the relatively high doses of UA required to achieve weight reduction can result in serious side-effects. For example, Food and Drug Administration (FDA) received at least 21 reports of hepatotoxicity in consumers who ingested dietary supplements containing UA or sodium usniate for weight loss, thereby raising safety concerns. These hepatotoxicities resulted in one death, one liver transplant, seven individuals with liver failure, 10 cases of chemical hepatitis, and four cases of mild hepatic toxicity[13-15]. Irrespective of a long history of using UA containing products, only a few animal studies were conducted to evaluate its clinical safety[16]. Pharmacokinetic

studies on rabbits have proved that single oral dose of UA (20 mg/kg) shows no evident signs of toxicity[17]. A study on larvae of herbivore insect (Spodoptera littoralis), which received injections of UA in the hemolymph, indicated that the (-)- form was found to be ten times more toxic than its (+)- form (LD_{50} 8.6 versus 90.8 µmol)[18].

The present study was undertaken in order to evaluate the plasma level of UA treated-rats, following oral administration of single UA dose (500 mg/kg). As well as to determine the safety dose based on the biological effect of chronic oral administration of 2 doses of UA (100 and 300 mg/kg) for 7 weeks, n-hexane extract of *Usnea articulata*, on body weight, body mass index (BMI), food and water uptake, liver and perirenal adipose tissue indices in adult male Sprague-Dawley (SD) rats. The changes of plasma levels of glucose, insulin, magnesium, liver function tests, lipid profile, leptin, glutathione were also recorded and compared.

Methods and Procedures

The prospective experimental study was conducted at King Fahad Medical Research Center (KFMRC) and the Biochemistry Laboratory of King Abdulaziz University Hospital (KAUH), Jeddah, Saudi Arabia.

(+)-*Usnic acid 98%*

(+)-Usnic acid (Molecular Weight: 344.32, Assay: 98%, optical activity: [α]25/D +488°, c = 0.4 in chloroform, mp: 201-203°C) prepared from *Usnea dasypoga*, 2(6-Diacetyl-7), 9-dihydroxy-8, 9*b*-dimethyldibenzofuran-1, 3 (2*H*,9*bH*) dione ($C_{18}H_{16}O_7$) was purchased from Sigma-Aldrich Chemie Gmbh, Munich, Germany.

Plant

Usnea articulata was collected from Asir region in the southwestern of Saudi Arabia, where it is growing on Juniper trees (Juniperus procera) in Al-Sawdah ("Jabal Sawdah" 18º20'N, 42º18' E). The plant was previously identified[4,19].

Extraction and Isolation of UA

Air-dried pieces (2500 g) of *Usnea articulata* were soaked for 5 days in dark vials containing 2500 ml of n-hexane on an orbital shaker. Three time filtration was carried out using Celite® poured on Whatman filter

paper. The solid residues were discarded and the crude extract was re-filtered with Whatman filter paper. The solvent (*n*-hexane) was removed by evaporation at 69º using a Heidolph Rotary Evaporator, Laborota 4000 (Sigma-Aldrich Chemie Gmbh, Munich, Germany). The concentrated crude extract was dissolved in absolute ethanol with a stirrer in a water bath. The filtrate was kept overnight at 0-4ºC. Long yellowish crystalline prisms of usnic acid[19] was purified by recrystallization in ethanol, weighed to calculate its percentage then kept in stopper vials at room temperature.

Animals

Adult male SD rats (n = 37) obtained from the animal house unit in KFMRC were used. This study consists of two experiments. The animals used were housed in plastic cages (42 x 26.5 x 15 cm) at 20ºC and 60% humidity with 12/12 hr dark-light cycle with free access to water and commercial rat food. Animals were acclimatized for one week before starting the experiments. The experimental procedures were carried out according to "Guideline on Experiments on Animal" at KFMRC, King Abdulaziz University and approved by Ethical Committee.

Experimental Protocols

Experiment (I)

This experiment aimed to determinate UA in rat plasma by using modified method[20] *via* high pressure liquid chromatography (HPLC). The HPLC system consisted of an Alliance Waters separation module 2695 and a photodiode array detector model 2996 (Milford, MA, USA). The column heater was set to 25 ± 2°C. The control of the HPLC system and data processing were performed with Empower Software (Build 1154, Waters Corp., Milford, MA, USA).

A total of 7 adult male SD rats, weighing 300 gram each was caged individually (one/cage). A single dose of (+)-Usnic acid (500 mg/kg) suspended in 1 ml of 1% carboxymethylcellulose (CMC) -water solution was gavages to the animals. After 1, 3, 6, 12, 24, 48, and 72 hr, 1.5 ml of blood was collected *via* orbital puncture[21] in an ethylenediaminetetraacetic acid (EDTA) tubes and centrifuged for 15 min at 1100 × *g*. Plasma was separated carefully and stored at –70°C

until the analysis of UA for determination the time of maximal levels of UA in plasma.

Experiment (II)

The aim of this experiment was to determine the effect of low and high doses of UA on water and food intake, weight gain and metabolic parameters in the plasma in addition to morphology of perirenal adipose tissue.

Thirty adult male rats (weighing 150–218 gram) were divided into 3 groups (10 rats each). The rats were caged individually (one/cage). The animals were treated as follows:

Group (1): control group, administered 1 ml of 1% CMC-water solution (low viscosity).

Group (2): low dose treated group, administered 1 ml of 100 mg/kg UA (*U. articulate* extract) suspended in CMC.

Group (3): high dose treated group, administered 1 ml of 300 mg UA/kg suspended in CMC. All treatments were given 5 days per week for 7 weeks. Body weight, length (using a portable electronic digital scale), food and water intake were recorded weekly. Animals were monitored daily for abnormal clinical signs. Food efficiency (%) was calculated according to the formula: (final body weight gain / total food intake) × 100[22]. Body mass index (BMI) was calculated according to the formula: body weight (grams) / square of the height (centimeters)[23]. At the end of experiment, rats of all groups were overnight fasted and between 07:00 and 09:00, deeply anesthetized with ether[21], and blood was collected *via* periorbital venous plexus in an EDTA tubes and centrifuged for 15 min at 1100 × *g*. Plasma was separated and stored at –70°C for further biochemical studies. The animals were sacrificed by cervical dislocation and the abdomen was opened; liver and perirenal fat were removed and weighed. Organ and tissue indices [**(**liver or adipose tissue weight **/** body weight**)** X 100][24] were calculated. According to Ross *et al.*[25] adipose tissue was fixed in 10% neutral buffered formalin and further processed for light microscopic study. Paraffin sections (5 µm thick) were stained with hematoxylin and eosin (H&E), and examined by Olympus BX-51 light microscopy (Japan)**.** Adipocytes were photographed. Adipocytes number and area were studied using the

software Image-Pro plus version 6 analyzer. Notice: The scale bars of all photographs were standardized depending on actual magnification**.**

Biochemical Analysis

The plasma level of glucose, magnesium, total protein, albumin, total bilirubin, alkaline phosphatase test (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gammaglutamyltransferase (GGT); as well as lactate dehydrogenase (LDH), triglycerides (TG), cholesterol (CHOL), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein-cholesterol (HDL-C) were determined by enzymatic methods on an automated chemical analyzer (Dimension® RxL Max® Clinical Chemistry System, USA). Plasma level of leptin was measured spectrophotometrically using Rat Leptin 96-Well Plate Assay ELISA kit purchased from Linco Research, Inc., St. Charles, MO USA. The sensitivity of the kit was 0.04 ng/ml. The intra-assay and inter-assay variations of the kit were 2.13-2.49 and 2.95-3.93, respectively. Insulin plasma level was estimated spectrophotometrically using DRG® Ultrasensitive Rat Insulin ELISA (EIA-2943) purchased from DRG International Inc., Mountainside, NJ USA. The sensitivity of the kit was 1.76µIU/ml. The intra- and inter-assay variations of the kit were 17.45-66.43 and 17.36-66.90, respectively. The degree of insulin resistance was measured by the homeostasis model assessment of insulin resistance (HOMA-IR), which was calculated with the formula: Fasting serum insulin (µU/ml) X fasting plasma glucose (mmol/L)/ 22.5[26]. Determination of glutathione plasma levels was done using QuantiChrom™ Glutathione Assay Kit (DIGT-250) purchased from BioAssay Systems, Hayward, CA USA. The sensitivity and accuracy of the kit ranged from 0.4 - 100 µM.

Statistical Analysis

Statistical analysis of the data was performed using the Statistical Package for Social Science (SPSS) version 12. The data are expressed as means +/- standard error (SE). Comparison of variables between groups was performed using one-way analysis of variance (ANOVA) and "student's" *t* test as appropriate. The least significance difference test was employed to compare means for pairs of groups. All analyses with a p-value < 0.05 were considered statistically significant.

Results

Usnea articulata collected from Al-Sawdah Mountain, Asir region, Saudi Arabia, was found to contain 0.16% ± 0.03 UA crystals (specific rotation, [α] 25/D + 500° in chloroform; melting point, 198.9–199.1°C). Following a single UA oral dose of 500 mg/kg., UA level was found in rat plasma at one hr and 72 hr were 20.88 ± 2.63 µg/ml and 3.31 ± 2.18 µg/ml, respectively. The maximum concentration of UA in the plasma was obtained at 6 hr after administration (70.68 ± 9.12 µg/ml) (Table 1).

Table 1. Usnic acid (µg/ml) in rat's plasma at different hours following oral administration of a single dose (500 mg/kg).

Time (hr) after Oral Administration	**UA Concentration** (µg/ml) (mean ± SE)
1st	20.88 ± 2.63
3rd	55.59 ± 6.79
6th	70.68 ± 9.12
12th	52.06 ± 7.75
24th	39.17 ± 12.98
48th	17.75 ± 9.30
72th	3.31 ± 2.18

Although three rats of group (3) died within 5-7 weeks from the start of experiment, the results showed that at the end of the 1st week there was an insignificant increase in the percentage body weight gain after administration of low dose UA (11.42 ± 0.95% *vs.* 11.57 ± 0.73%, $p > 0.05$) compared to control group. However, there was an insignificant decrease in percentage body weight gain (11.42 ± 0.95% *vs*. 11.26 ± 0.97%, $p > 0.05$) after high dose (300 mg/kg). Meanwhile, from the second week to the end of the 7th week, there was insignificant decrease in the percentage body weight gain in all groups receiving low and high doses of UA compared with the controls. Regarding food intake, there was significant increase in food intake at 2nd, 4th and 5th weeks in G3 compared with controls in the same week ($p < 0.05$). Water intake increased significantly in G2 at 1st, 4th, 5th weeks versus control ($p < 0.05$), meanwhile, in G3 it was significantly increased in the 3rd, 4th, 5th, 7th weeks versus G2 and controls ($p < 0.05$) (Table 2).

Comparing the initial (beginning of 1st week) to final (end of the 7Th week) BMI, there was significant increase in G1 (controls) and G2 ($p <$

0.05) meanwhile, no significant changes were found in G3 ($p > 0.05$) (Table 3).

At the end of the 7^{th} week, food efficiency (%) was increased insignificantly in G2, whereas it was decreased significantly in G3 compared with G2 and control ($p < 0.05$). In G2, plasma levels of albumin, total bilirubin, cholesterol and HDL-C were significantly increased compared with controls (G1) ($p < 0.05$). Meanwhile, in G3 plasma levels of glucose, magnesium, total bilirubin, alanine aminotransferase, cholesterol, low density lipoprotein cholesterol, and high density lipoprotein cholesterol and liver index were significantly increased ($p < 0.05$) while; lactate dehydrogenase were significantly decreased than controls and G2 ($p < 0.05$). In G3, aspartate aminotransferase was significantly decreased than controls ($p < 0.05$) (Table 4).

Table 2. Effect of oral usnic acid (UA) given for 7th weeks on body weight gain, food and water intake.

Parameters	Weeks						
	1st	2nd	3rd	4th	5th	6th	7th
Body weight gain (%)							
Group (1) (0 mg UA/kg)	11.42±0.95	26.04±1.84	44.34±5.70	49.61±3.17	58.67±3.60	64.82±4.25	70.24±4.50
Group (2) (100 mg UA/kg)	11.57±0.73	25.41±2.09	37.11±3.07	46.38±3.65	53.20±4.77	59.90±5.291	65.17±5.58
Group (3) (300 mg UA/kg)	11.26± 0.97	24.62±1.27	35.68±1.28	44.55±2.36	50.00±2.07	53.58±2.62	60.98±2.84
Food intake (grams)							
Group (1) (0 mg UA/kg)	119.75±3.22	134.31±5.056	142.14±5.76	142.84±4.58	136.72±2.98	141.33±4.84	132.91±5.47
Group (2) (100 mg UA/kg)	135.83±7.79	150.49±4.51	144.89±5.10	150.16±3.84	142.8±4.76	150.62±4.56	145.02±6.09
Group (3) (300 mg UA/kg)	129.7±6.61	**151.41±7.46***	157.99±7.86	**165.09±8.79***	**153.29±6.85***	136.26±13.00	146.83±5.16
Water intake (ml)							
Group (1) (0 mg UA/kg)	123.20±2.18	152.10±3.85	145.20±2.09	144.30±2.16	140.60±2.32	145.10±3.97	142.90±3.16
Group (2) (100 mg UA/kg)	**136.40±4.96***	156.70±4.27	150.10±4.28	**156.80±3.35***	**152.90±2.62***	149.60±5.88	148.30±5.39
Group (3) (300 mg UA/kg)	130.70±4.96	163.60±5.32	**160.80±3.68*¥**	**167.40±3.56*¥**	**169.90±3.46*¥**	154.78±11.06	**178.43±10.02*¥**

*Data are expressed as mean +/- SE, * Significance versus controls, ¥ significance versus group (2) of same week. P< 0.05*

Table 3. Effect of different doses of usnic acid (UA) on body mass index (BMI) in different groups.

BMI (gram/cm^2)	**Group (1)** (0 mg UA/kg)	**Group (2)** (100 mg UA/kg)	**Group (3)** (300 mg UA/kg)
Initial (1st week)	0.156 ± 0.001	0.156 ± 0.003	0.156 ± 0.004
Final (7th week)	0.190 ± 0.003	0.190 ± 0.006	0.173 ± 0.01
% changes from initial to final	**21.19 ± 2.06***	**21.41 ± 2.330***	10.33 ± 5.34

Data were expressed as mean +/- SE. **Significance initial versus final BMI of the same group. P < 0.05*

Table 4. The effect of usnic acid (UA) administration (100 or 300 mg/ kg) given five times a week for seven weeks on different measured parameters at the end of the 7th week.

Parameters	**Group (1)** (0 mg UA/kg)	**Group (2)** (100 mg UA/kg)	**Group (3)** (300 mg UA/kg)
Food efficiency (%)	0.13±0.30	0.27±0.21	**– 0.71±0.30**$^{*¥}$
Glucose (mmol/L)	6.66±0.34	7.40±0.43	**9.00±0.60**$^{*¥}$
Insulin (µg/L)	0.76 ± 0.12	0.74 ± 0.12	0.54 ± 0.13
Insulin resistance	6.20±1.07	7.31±1.60	6.18±1.64
Magnesium (mmol/L)	1.01±0.04	1.07±0.04	**1.34±0.05**$^{*¥}$
Total protein (g/L)	62.4±0.65	65.80 ±0.84	66.88±3.00
Albumin (g/L)	13.8±0.20	**14.90±0.23***	14.13±0.64
Total bilirubin (µmol/L)	2.20±0.13	**3.00±0.15***	**5.00±0.50**$^{*¥}$
Alkaline phosphatase (µ/L)	125.7±7.03	115.10±5.04	117.0±11.16
Aspartate aminotransferase (µ/L)	89.3±3.36	86.30±3.10	**78.00±1.36***
Alanine aminotransferase (µ/L)	54.5±2.33	57.50±1.92	**74.25±6.07**$^{*¥}$
g-Glutamyl transferase (µ/L)	2.22±0.28	2.00±0.41	2.71±0.57
Lactate dehydrogenase (µ/L)	676.9±84.78	599.30±63.27	**340.88±39.23**$^{*¥}$
Triglycerides (mmol/L)	0.62 ± 0.10	0.77 ± 0.12	0.66 ± 0.07
Cholesterol (mmol/L)	1.76 ± 0.07	**2.11 ± 0.10***	**2.62 ±0.14**$^{*¥}$
Low density lipoprotein cholesterol (mmol/L)	0.27 ± 0.02	0.31 ± 0.02	**0.39 ± 0.03**$^{*¥}$
High density lipoprotein cholesterol (mmol/L)	0.53 ± 0.01	**0.64 ± 0.03***	**0.79 ± 0.05**$^{*¥}$
Leptin (ng/ml)	1.78 ± 0.79	1.49 ± 0.61	0.26 ± 0.13
Glutathione (µM)	157.79 ± 4.00	155.46 ± 5.92	146.57 ± 9.05
Liver index (%)	3.03 ± 0.08	2.92 ± 0.09	**3.39 ± 0.10**$^{*¥}$
Perirenal adipose tissue index (%)	1.18 ± 0.21	1.29 ± 0.23	0.87 ± 0.15
Adipocyte number/ 40.000 µm^2	48 ± 3.27	51.5 ± 3.89	61.94 ± 6.33
Adipocyte area (µm^2)/ 40.000 µm^2	1186.11 ± 76.98	1136.97 ± 89.30	1015.81 ± 101.58

Data were expressed as mean +/- SE. **Significance versus controls;* $^{¥}$ *significance versus group (2). P < 0.05*

Histological Studies

In H&E stained sections, adipocytes of perirenal fat of G1 (control) have the usually signet ring appearance described in literature. In animals receiving CMC they did not exhibit any significant morphological or morphometric changes (Fig. 1). Adipocytes of G2 (100 mg UA/kg) showed slight decrease in cell area (Fig. 2) with concomitant increase in cell number per field (40,000 μm^2) ($p > 0.05$). In G3 (300 mg UA/kg), adipocytes looked deformed with interspacing congested vessels or hemorrhage (Fig. 3). The area was insignificantly decreased while the cell number showed insignificant increase ($p > 0.05$) (Table 4).

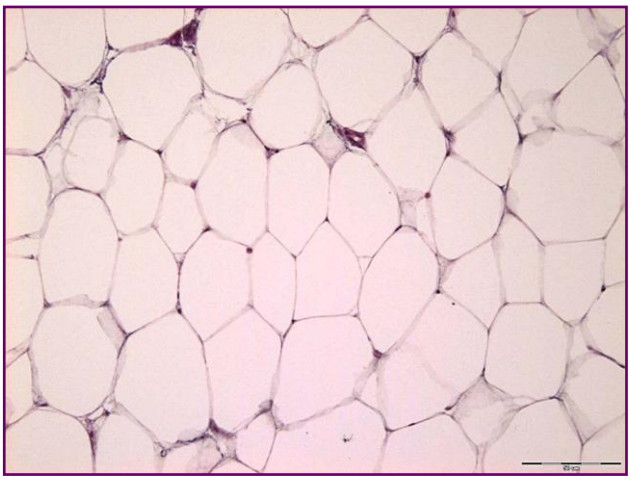

Fig. 1. Adipocytes of rat perirenal adipose tissue after administration of vehicle [1% carboxymethylcellulose (CMC)] (control) showing normally shaped adipocytes. (H&E: X 200).

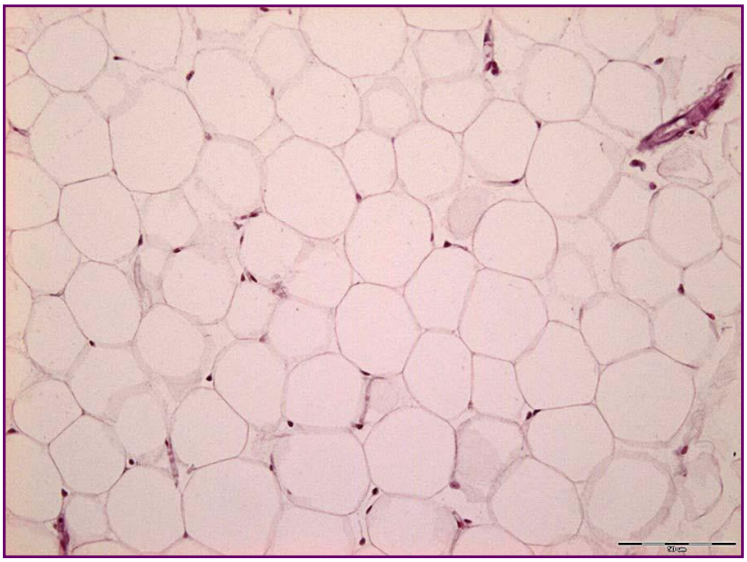

Fig. 2. **Adipocytes of rat perirenal adipose tissue (G2) after administration of UA (100 mg/kg). The cells look smaller than in the control group. There are no signs of cell necrosis, fibrosis, or vascular changes. (H&E: X 200).**

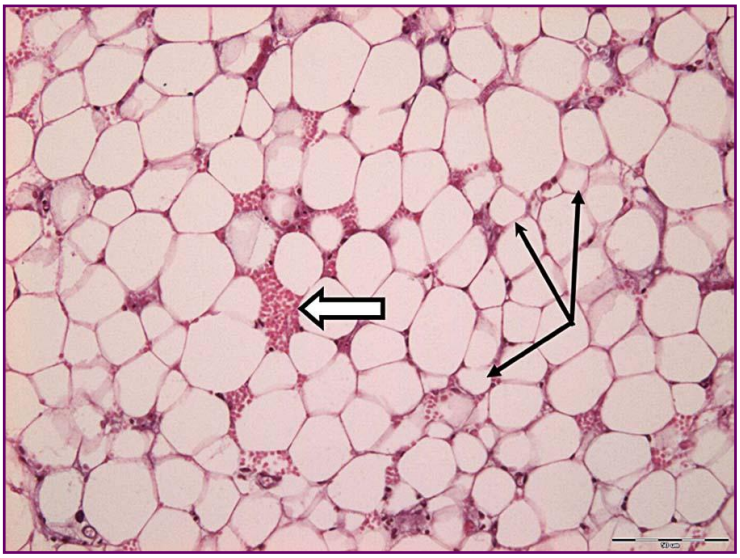

Fig. 3. **Adipocytes of a rat perirenal adipose tissue (G3) after administration of UA (300 mg/kg). Significant deformity and decrease in cell size (may indicate cell apoptosis) of most adipocytes (black arrows) are observed following 8 weeks of UA administration. Notice the marked capillary congestion and associated intercellular hemorrhage (white arrow). (H&E: X 200).**

Discussion

The public's increasing demand for alternative medicine and the newly found global interest in phytomedicine and herbal therapies have led to a rapid rise in the use of unregulated herbal supplements and therapies. Usnic acid, extracted from lichen species, was recently included in some lipolytic products (LipoKinetix) with contradictory data concerning its hepatotoxicity[13,15].

Pharmacokinetic studies of standard UA performed herein showed that it was well absorbed from the digestive tract of rats reaching a concentration peak (70.68 μg/ml) in plasma within 6 hrs after oral administration (500 mg UA/kg). Furthermore, the results indicated that UA remained in the circulation for a period of 72 hrs (3.31 μg/ ml). A peak of 32.5 μg/ml was found in rabbit plasma 12.2 hr following oral administration of UA in a dose 20 mg/kg[17].

Experimental animals in this study appeared healthy and showed normal activity with no signs of stress at either dose used orally (100 and 300 mg/kg), which revealed the potential safety especially at low doses. However, in previous study on mouse, UA from *Usnea articulata* was reported to have a median lethal dose of 180 mg/kg to mice (s.c. injection), and symptoms of UA toxicity was similar to those of classical oxidative phosphorylation uncouplers[27].

In rats receiving low dose of UA (100 mg/kg), the results of this study showed significant increase in BMI at the 7th week compared to 1st week. There is no available data on the effect of extracted UA (without additives) on body weight in animals, despite potential use of UA in alternative medicine as a commercial health-promoting product[2,15].

At the 7th week, the liver index (%) of lean rats treated with high dose (300 mg/kg) UA was increased significantly ($p < 0.05$), while perirenal adipose tissue index (%) showed an insignificant decrease. These results could be explained in view of the reports that mobilization of stored lipids in cases of ATP deficiency (could be here due to thermogenic effect of UA) was reported to associate with subsequent deposition within liver parenchyma[28]. On the other hand, insignificant changes in liver index (%) and perirenal adipose tissue index (%) were observed in rats received low dose of UA (100 mg/kg).

Loss of membrane integrity or altered permeability due to lack of ATP[29] with subsequent leakage of cytoplasmic contents resulting in

increased serum liver enzymes including alanine aminotransferase was reported in literature[28,30]. Similar suggestion could be given here to explain the significant increase of alanine aminotransferase associated with high dose of UA. The (+) UA hepatotoxic effect was presumably a result of mitochondrial dysfunction[2,29,31,32]. Future work concerning hepatocyte ultrastructure is going on to confirm such suggestion.

The release of lactate dehydrogenase and aspartate aminotransferase is commonly used as an indicator of plasma-membrane damage[33]. However, these enzymes were significantly decreased in animals received high dose of UA than controls. This result could not be explained in the present study. In contrast, UA, at 323 mg/kg/day, was reported to produce a transient elevation of serum lactate dehydrogenase and aspartate aminotransferase in 1 out of 9 domestic sheep[34].

Moreover, a significant change in serum level of aspartate aminotransferase and alanine aminotransferase was observed when rats received high doses of (+) UA (200 mg/kg per day, i.p. for 5 days)[16]. A species-dependent effect could underlie such differences. Also, UA at high dose (1 mM) induced a loss of cell membrane integrity in isolated rat hepatocytes, which was detected by the release of cellular transaminases (AST and ALT) into the culture media[16]. In clinical field, two patients taking a commercial weight loss product, containing UA and soybean, were reported to have elevated serum levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and total bilirubin[35]. Several hepatotoxicity cases experienced by several individuals who used an herbal preparation called LipoKinetix. This commercial preparation contained norephedrine hydrochloride, sodium usniate, 3,5-diiodothyronin, Yohimbine Hydrochloride and caffeine[13]. On the other hand, the specific syndrome observed in lichen-poisoned animals would seem to indicate that other compounds in the lichen, possibly in addition to (+) UA, interact to produce such toxicity[34].

In this study, total bilirubin was significantly increased in rats received low and high doses of UA (100 and 300 mg/kg); while albumin was significantly increased in animals received low dose of UA compared to controls. The increase in total bilirubin reported in this study in animals receiving both low and high doses of UA (100 or 300 mg/kg) could be due to impaired conjugation of bilirubin[36]. Altered rough endoplasmic reticulum, mitochondrial function, and lack of ATP resulting from uncoupling of oxidative phosphorylation by UA[16,29,31] could also

predispose to such impairment. Serum total protein and albumin changes may aid in diagnosing chronic or inflammatory liver diseases[37].

Estimation of serum lipid levels showed that cholesterol and HDL-C were significantly increased in rats treated with low and high doses with UA, while LDL-C was significantly increased in high dose UA treated group compared with controls. An increase in serum cholesterol was logical as both HDL-C and LDL-C were increased in experimental groups. HDL-C was designated as a "good" cholesterol carrier and an inverse relationship was reported between plasma HDL-C concentration and atherosclerosis[38]. The slight increase in triglyceride was most probably due to the mobilization of stored fat in adipose tissue. A fat burner agent (*e.g.,* green tea) was reported to be associated with increased triglyceride serum level[39]. However, there are no available studies concerning such an effect with UA.

Usnic acid (UA) was proved by Hsu *et al*.[35] to be a fat burner. Its effect on adipocyte in the present study was observed in the form of decreasing cell area and number in low dose animal group, while high dose result in cell necrosis of both adipocyte and endothelial cells resulting in intercellular hemorrhage. No available literature describes similar effect on adipocytes. However, cell necrosis rather than apoptosis was described by Einarsdottir *et al.,*[40] and Backorova *et al.,*[41] to be the mechanism of anti-cancer effect of lichen compound usnic acid. Adipocyte atrophy or necrosis described herein could be also explained in view of actions of other fat burners such as CLA. It was found that *cis*-9, *trans*-11 CLA has differential effects on lipid metabolism[42]. The same authors found that cultures treated with 50 mol/L *trans*-10 and *cis*-12 CLA, showed an increase in basal lipolysis by 18% compared with controls in 3T3-L1 preadipocytes. A recent study showed that oxidative phosphorylation and gluconeogenesis were dramatically inhibited by 10μM UA[43]. A similar mechanism could be evoked in adipocytes result in depletion of ATP, decreases storage function and concomitant cell atrophy or death. Reviewing available literature, the present study could be the first that describe the effect of UA on adiposity and lipid profile at preclinical level.

The increase in food intake, decrease in food efficiency associated with low serum insulin level might have a role in the significant increase in serum glucose level in high dose. Uncoupling of oxidative phosphorylation with a consequent possible defect in energy

transformation pathways could underlie such an increase[44]. Pancreatic insulin secretion was reported to be directly proportional to the size of the fat mass[45]. The present study could be preliminary as there is no available literature concerning such effect.

The significant increase in magnesium observed in this study in the high dose group (300 mg UA/kg) could be due to uncoupling of oxidative phosphorylation. Studies on activated cultured hepatocytes have shown that glucokinase binds to the cell matrix by a Mg-dependent mechanism such as mitochondrial Mg^{2+}-activated ATPase[46]. Thus, there is a possibility that uncoupling of oxidative phosphorylation by UA disrupts this binding, and therefore, releases Mg^{2+} into the circulation, resulting in an increase of its level in serum.

From the present results it could be suggested that UA has no effect on central regulation of food intake based on the observation of increasing food intake; which herein was found to be associated with slight reduction in perirenal adipose tissue index and serum leptin level. Leptin from adipocyte source[47] has a well-known action in regulating eating behavior[48] *via* stimulation of lateral and ventromedial hypothalamic nuclei with subsequent suppression of food intake[49].

Glutathione is the strong nucleophilic molecules found in most cells with its function as an antioxidant[16]. A slight decrease in serum glutathione was observed in rats receiving a high dose of UA (300 mg/kg), which could be attributed to an oxidative stress[31]. Usnic acid (25, 50, 100 and 200 mg/kg) was found to increase glutathione levels in rat, which was reduced by indomethacin[50]. A decrease in glutathione content was described in isolated rat hepatocytes after treatment with a high dose of UA (1 mM)[16]. The same authors identified a directly hepatotoxic effect of usnic acid on isolated rat hepatocytes in a mechanism similar to carbon tetrachloride, which involves free radical generation with resultant cell membrane and mitochondrial injury[51], lipid peroxidation, disturbed calcium homeostasis, and cell death.

In conclusion, UA could be considered potentially safe in low dose. It was found to have a dose-dependent effect on body weight, liver and perirenal adipose tissue indices, liver enzymes, and lipid profile and blood glucose. Hepatotoxic effect of (+) usnic acid could be direct or by its reactive metabolite(s) causing loss of membrane integrity. Usnia acid extracted from *Usnea articulate* could be considered quietly safe if used

orally in low doses as it showed minimal or insignificant effects on most parameters tested in this study. More studies are also needed to test such safety in obese animals as a weight-reducing agent. Adjustment of UA dose and imply nanotechnology as nano encapsulation in microspheres could be used in the future for safe preparation of UA formulations targeting only fat stores.

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تقييم سلامة حمض الأوزنيك (مستخلص أشنة أوزنيا أرتيكيولاتا) على صورة الدهون، شكل الخلايا الدهنية ووظائف الكبد في ذكور الجرذان

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المستخلص تهدف الدراسة إلى تقدير سلامة المعالجة بحمض الأوزنيك (أشنة أوزنيا أرتيكيولاتا) عند الجرذان. تم تحديد معدل الامتصاص وأعلى تركيز لحمض الأوزنيك في البلازما. استخدم في الدراسة ثلاثة مجاميع (١٠ جرذان لكل مجموعة). مجموعة () عولجت بمادة كربوكسي ميثيل سيليلوز ١٪، المجموعتان (٢،٣) عولجتبالمستخلص بجرعات (١٠٠، ٣٠٠ ملجم/كجم) على التوالي بالفم ٥ أيام في الأسبوع لمدة ٧ أسابيع. وتم جمع عينات الدم للتحاليل الكيموحيوية. تم فحص النسيج الدهني مجهريا. بعد تجريع ٥٠٠ ملجم/كجم سجل أقصي تركيز بالدم بعد ٦ ساعات وظل في الدورة الدموية٧٢ ساعه. في الأسبوع السابع لوحظ في المجموعة (٢) زيادة في كل من الألبيومين والبيليروبين الكلي والكوليستيرول الكلي والكوليستسرول البروتيني الدهني عالي الكثافة. أظهرت المجموعة (٣) زيادة في كل من الجلوكوز والمغنيسيوم والبيليروبين الكلي والالنين أمينوترانزفيريز والكولستيرول الكلي والكوليستيرول البروتيني الدهني عالي الكثافة ومعامل الكبد، بينما انخفض فيها كل من أسبارتيت

أمينوترانزفيريز ، ولاكتيت ديهايدروجينيز . زادت في المجموعة٢() الكفاءة الغذائية وإنزيم لاكتيت ديهايدروجينيز وانخفض كل من الجلوكوز والمغنيسيوم والبيليروبين الكلي وإنزيم الانين أمينوترانزفيريز والكولستيرول الكلي والكوليستيرول البروتيني الدهني العالي والمنخفض الكثافة ومعامل الكبد مقارنة بالمجموعة (٣). لمستخلص الأشنة تأثير بيولوجي على الجرذان اعتماداً على الجرعة المعطاة ، مع مستوى آمن للجرعة المنخفضة. كما وجد أن له تأثير على كل من شكل وحجم الخلايا الدهنية خاصة في حالة الجرعة المرتفعة