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Myrica rubra Fruit Drink Sub-Chronic Toxicity and Hepatoprotective Effect in Rats

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Abstract

Background: This study dealt with the effect of the subchronic toxicity of *Myrica rubra* fruit beverage drink (MRD) in rats and its hepatoprotective effect against carbon tetrachloride (CCl_4)-induced hepatotoxicity.

Methodology: Different groups of normal male and female Wistar rats were treated with 50% MRD as drinking vehicle (13 weeks), as substitution of the normal drinking water. Coulter Counter was used for red blood corpuscles (RBCs) and white blood corpuscles (WBCs) count. The Reflotron instrument and Reflotron haemoglobin kit used for determination of haemoglobin content, while the Reflotron strips for determination of blood glucose, total triglycerides and cholesterol contents, blood enzymatic levels, and bilirubin. Atomic absorption spectroscopy was used for determination of blood Na^+, Mg^{++} and Ca^{++} concentrations.

Principal findings: Treatment induced significant increases in the red blood corpuscles (RBCs) count, haematocrit and haemoglobin content. It also significantly decreased plasma levels of total cholesterol and the low-density lipoproteins (LDL) without affecting the levels of high-density lipoproteins (HDL), glucose, triglycerides and bilirubin, together with the significant decrease in hepatic malonaldehyde production. The treatment resulted in significant reductions in the enzymes alanine transiminase (ALT), aspartate aminotransferase (AST) and Alkaline phosphatase (ALP) and a significant diuretic effect.

Conclusion: The results of the study point to the potential of *Myrica rubra* fruit drink to act as a new functional food.

Keywords: *Myrica rubra* drink; Hepatoprotection; Hepatotoxicity; Hepatic enzymes

Introduction

Myrica rubra or Bayberry, Yumberry, Waxberry or Chinese strawberry tree is usually cited as *Myrica rubra* by Siebold and Zuccarini (1846).

Myrica rubra sieb et zucc. fruits, family Myricaceae, are stony red fruits with berry-like edible portions. The fruit is grown in China, India, Japan and some other south eastern Asian countries such as, Vietnam, Burma and Thailand. It is known by various other names such as Yangmei, Bayberry and Chinese arbutus. It is also known as Wax-mytle and Yamamoto in Japan.

Phytochemical investigations of the fruit juice revealed the presence of high concentrations of polyphenols and proanthocyanidins [1]. The latter are condensed tannins (polymers) composed of various flavan-3-ol or catechin units. The most available are the reddish procyanidins [2]. In addition to the fruits both of the leaves and the bark of the tree constituents were analyzed [3-5].

The proanthocyanidins were reported to possess various actions that included antioxidant [6,7], anti-viral [7,8], hypolipidemic [9], anti-cancer [10], and anti-inflammatory actions [11].

Myrica rubra juice is now widely distributed world-wise as a 50% refreshing drink and also as a carbonated beverage - under the trade name Yumberry. Thus, it was thought of interest to investigate the outcomes of the sub-chronic toxicity (treatment for 13 weeks) of this refreshing drink. When it was observed that it decreases some hepatic enzymes, it was thought to investigate the effect of short term treatment of rats (4 weeks) with the drink as a sole source of drinking vehicle.

Materials and Methods

Yumberry juice drink

Yumberry juice bottled drink was purchased from the local market in Riyadh city, Kingdom of Saudi Arabia. The bottled drink is a product of China (Zhejiang Yumberry Juice Co.).

Animals

In this study male Wistar rats (body weight 250 ± 10 g) and females (220 ± 8 g) were used. The animals were provided with standard chow diet, supplied by Silo and Flour Mills Organization, Feed Mill, Riyadh, Saudi Arabia. All animals were housed at a temperature of 22° ± 1°C and relative humidity of 50 ± 5%. The light: dark cycle was 12 hours each. The animals' treatment was conducted in accordance with the Guide for the Care and Use of Laboratory Animals. The protocol of the current study was approved by the ethics Committee of the College of Pharmacy, King Saud University, Riyadh, KSA.

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Treatment of the animals

The control group was allowed tap water ad lib. The treatment group was administered *Myrica rubra* juice refreshing drink (50% Yumberry drink) as the sole source of drinking vehicle for 13 weeks in case of the sub-chronic toxicity and for 4 weeks in the case of the Hepatoprotective activity.

To investigate the hepatoprotective effects, carbon tetrachloride CCl_4 was used as an inducer of hepatotoxicity. For this purpose, it was administered as a mixture of CCl_4: paraffin oil in the ratio of 1:1 at a dose of 1 ml/kg intraperitoneally (i.p). Male Wistar rats were divided into 3 groups (N=8 animals per group). Group No. 1 was injected with paraffin oil only (1 ml/kg intraperitoneally) as a single dose. Group 2 was injected with the mixture of CCl_4 and paraffin oil. Group 3-the one given Yumberry drink for 4 weeks-was administered the mixture of CCl_4 and paraffin oil as a single dose (1 ml/kg i.p.) in the morning following the 4-week-treatment period. Blood was collected on the third day.

Collection of blood from the sub-chronically-treated and control animals

On days 90 and 91 following the start of the treatment, the animals were anaesthetized with diethyl ether and blood (7 ml) was collected from each control and treated animal using cardiac puncture employing a 23-gange needle fitted to a 10-ml plastic syringe. 1 ml from each animal was used for determination of the various blood cell counts, the hemoglobin content and the clotting times. The remaining blood (6 ml each) was mixed with 3.6 (w/v) aqueous trisodium citrate solution in the ratio of 1:9 (citrate:blood) to prevent blood clotting. The blood was then centrifuged at 3000 rpm (EMS Centrifuge) for 20 minutes to obtain platelets and blood cells-free plasma and to calculate the haematocrit value for each animal. The latter were corrected for the added trisodium citrate. The plasma was then stored at -20°C until used within the next few days for the determination of the various parameters indicated below.

Determination of blood cell counts

The numbers of RBCs and WBCs per µl whole blood obtained from each control or treated rat was determined using Coulter Counter, Model S8 90 (Coulter Electronics, Lubon beds, U.K.). The volume of blood used was 125 µl/test.

Determination of haemoglobin content

The haemoglobin content was determined using the Reflotron Instrument and Reflotron haemoglobin kit (Roche Diagnostic GmbH, D-68298, Mannheim, Germany). The test depends upon conversion of haemoglobin to methaemoglobin in presence of potassium ferricyanide as outlined by Van Assendelft [12].

Determination of the haematocrit value

The haematocrit value of each blood sample was calculated following the centrifugation of the collected blood in graduated centrifuge tubes at 3000 rpm (EMS Centrifuge) for 20 minutes.

Determination of blood glucose level

The concentration of glucose was determined in each cell-free plasma using the Reflotron Instrument and the Reflotron Glucose strip (Roche Diagnostics). It depends upon the principle of conversion of glucose in the sample in presence of molecular oxygen and the enzyme GOD (Glucose Oxidase Dehydrogenase to S-D-gluconolactone and H_2O_2. The latter in presence of the indicator 3,3,5,5-tetramethyl benzidine and the enzyme POD (peroxidase 'horseradish') gives a color which was measured as outlined by Trasch [13].

Determination of blood total cholesterol

The cholesterol contents in the different plasmas were determined using the Reflotron instrument and the Reflotron strips (Roche Diagnostics) depending upon the principle of conversion of the blood cholesterol esters to cholesterol in presence of the enzyme cholesterol esterase. The produced cholesterol is then converted in presence of molecular oxygen and cholesterol Oxidase to cholestenone and H_2O_2. The latter in presence of the indicator 3,3,5,5-tetramethyl benzidine and the enzyme POD (peroxidase 'horseradish') gives a color which was measured as outlined by Braun [14].

Determination of blood total triglycerides

The triglycerides contents of the different plasmas were determined using the Reflotron Instrument and the provided strips (Roche Diagnostics). The method depends upon the principle of conversion of the blood triglycerides in presence of the enzyme esterase to glycerin and fatty acids. The produced glycerin in presence of ATP and the enzyme glycerin kinase I is then converted to glycerin-3-phosphate and ADP. The glycerin-3-phosphate is then acted on by the enzyme glycerin phosphate Oxidase and molecular oxygen to produce dihydroxyacetone phosphate and H_2O_2. The latter in presence of the indicator 4-(4-dimethylaminophenyl) 5-methyl-2-(3,5-dimethoxy-4-hydroxyphenyl) imidazole dihydrochloride and the enzyme POD (Peroxide 'horse radish') produces a color which can be measured as outlined by Carstensen et al. [15].

Determination of blood GOT (AST) (Glutamic oxaloacetic acid transiminase or Aspartate aminotransferase)

The levels of AST in the different plasmas were determined using Reflotron Instrument and the provided strips (Roche Diagnostics, Germany). The principle depends upon the ability of the enzyme GOT to act on ketoglutarate and alanine sulfinate to produce glutamate and pyruvate. The latter in presence of molecular O_2, phosphate ions and water is acted on by the enzyme pyruvate Oxidase to CO_2, acetyl phosphate and H_2O_2. The latter in presence of the peroxidase enzyme and the indicator 4-(4-dimethyl aminophenyl) 5-methyl-2-(3, 5-di-t-butyl-4-hydroxyphenyl) imidazole dihydrochloride gives a blue color which intensity can be measured as outlined by Denake [16].

Determination of blood ALT (Alanine Transiminase) or GPT (Glutamate- Pyruvate Transiminase)

The concentrations of the ALT in the different plasmas were determined using the Reflotron Instrument and the provided strips (Roche Diagnostics, Germany). The principle depends upon the ability of ALT to convert ketoglutarate and alanine to glutamate and pyruvate. The latter in presence of PO_4^{2-}, water and molecular oxygen is then converted by the enzyme pyruvate Oxidase to acetyl phosphate, CO_2 and H_2O_2. The latter in presence of the enzyme peroxidase and the indicator 4-(4-dimethyl aminophenyl) 5-methyl-2-(3, 5-di-t-butyl-4-hydroxyphenyl) imidazole dihydrochloride produces a blue color, which color intensity, can be measured as outlined by Denake and Rittersdorf [17].

Determination of blood alkaline phosphatase

The contents of alkaline Phosphatase in the different plasmas were determined using Reflotron Instrument and the provided strips (Roche Diagnostics). The principle depends upon the ability of the enzyme alkaline Phosphatase to convert o-cresophthalein phosphate and methylglucamine to methylglucamine phosphate and the colored compound o-cresophthalein that can be measured at 567 nm as outlined by Rosalki and Heins [18,19].

Determination of blood K^+

The concentrations of K^+ in the different plasmas were determined using Reflotron Instrument and the provided strips (Roche Diagnostics). The principle depends upon the interaction and complexation of K^+ in the plasmas with valinomycin and the indicators 4-[(2,6-dibromo-4-nitrophenyl) azo]-2-naphthol to give a colored complex that can be measured as outlined by Lum and Cambizzino [20].

Determination of blood bilirubin

The concentrations of bilirubin in the different plasmas were determined using the Reflotron Instrument and provided strips (Roche Diagnostics). The principle depends upon the interaction of blood bilirubin with the indicator 2-methoxy-4-nitrophenyl diazonium tetrafluroborate to produce the colored product azobilirubin as outlined by Freitag [21].

Determination of blood Na^+, Mg^{++} and Ca^{++}

The concentrations of Na^+, Mg^{++} and Ca^{++} in the different plasmas were determined using atomic absorption spectroscopy using Varian AA775, atomic absorption spectrophotometer. For the determination of Na^+ the fixed working conditions were: Lamp current 5 mA, fuel: acetylene support air and flame stoichiometry: oxidizing. In the flame emission the wavelength used was 589.0 nm, the spectral band pass was 0.1 nm, the fuel: acetylene and the support: air.

For the determination of magnesium (Mg^{++}) the fixed working conditions were: lamp current 3.5 mA, fuel: acetylene, support: air and flame stoichiometry: oxidizing. The working flame emission conditions were: wavelength 285.2 nm, spectral band pass 0.1 nm, fuel: acetylene and support: nitrous oxide.

For the determination of Ca^{++}, the fixed working conditions were: lamp current 3.5 mA, fuel: acetylene, support: nitrous oxide and flame stoichiometry: reducing: red cone 1-1.5 cm high. The flame emission conditions were: wavelength 422.7 nm, spectral band pass 0.1 nm, fuel: acetylene and support: nitrous oxide [22,23].

Determination of urinary Na^+, K^+ and Ca^{++} contents

The concentrations of Na^+ and Ca^{++} in urine were determined using atomic absorption spectrophotometry as described above. The concentrations of K^+ were determined using Reflotron Instrument and specific strips as described above. It should be noted that following collection of urines, they were acidified immediately after collection by the addition of concentrated HCl (1 ml acid+3 ml urine) to prevent the precipitation of calcium oxalate and phosphate [24].

Statistical analysis

All values reported were the mean ± standard error (s.e.) of mean. Statistical differences were examined using ANOVA techniques using the least significant difference criterion or the '*t*' test as appropriate.

Results

General observations during the sub-chronic study regarding the general health of the animals

All animals treated with *Myrica rubra* drink (MRD) as the sole drinking vehicle for the whole 13 weeks were healthy. There were no any toxicological signs in the respiratory, cardiovascular, central and autonomic nervous system. There were no changes in the eyes.

Water and food consumption

Table 1 depicts the mean ± s.e. mean drinking vehicles and food/kg/week in the control and treated groups (males and females). The food and MRD consumed by the treated animals (both male and female rats) were significantly greater than that consumed by the corresponding control animals ($P<0.05$, $N=8$).

Effect on blood cells, haematocrit and haemoglobin

Table 2 depicts the effects of MRD treatment on total erythrocytes, leukocytes, platelets, haematocrit and haemoglobin content.

Effect on blood glucose, blood lipids and bilirubin

Treatment of both sexes of rats with MRD induced significant decreases in both total cholesterol and LDL ($P<0.01$, $N=8$) without any effect on glucose, HDL, triglycerides and bilirubin. Table 3 shows the cumulative results.

Effect on liver enzymes

Treatment of rats (both sexes) with MRD as the sole drinking

Animal group (Sex)	Mean Consumption/kg/week	
	Drinking Vehicle (ml)	Food (g)
Control (Male)	708 ± 17.3	541.3 ± 12.6
Control (Female)	661 ± 13.9	480 ± 16.3
MRD (Male)	879.9 ± 15.1*	639.5 ± 9.7*
MRD (Female)	830 ± 11.9*	576 ± 9.3*

*P<0.05, N=8, compared with the corresponding control.

Table 1: Water and Food Consumption by Rats during Treatment with MRD.

Treatment (sex)	No. of Cells/µl Blood			Haematocrit	Haemoglobin g %
	RBC×10^6	WBC×10^3	Platelets×10^3		
Control (male)	9.1 ± 0.07	13.6 ± 0.1	301 ± 0.2	43.5 ± 0.6	14.1 ± 0.1
Control (female)	7.8 ± 0.03	11.6 ± 0.08	285 ± 0.1	39.6 ± 0.2	13.5 ± 0.08
MRD (male)	10.08 ± 0.1*	13.9 ± 0.2	310 ± 0.3	48.3 ± 1*	15.7 ± 0.04*
MRD (female)	8.75 ± 0.07*	11.4 ± 0.1	291 ± 0.2	44.9 ± 0.7*	14.8 ± 0.05*

*P<0.01, N=8, compared with the corresponding control.

Table 2: The effects of MRD treatment on total erythrocytes, leukocytes, platelets, haematocrit and haemoglobin content.

Parameter	mg %					
Treatment (sex)	Glucose	Total Cholesterol	LDL	HDL	Triglyceride	Bilirubin
Control (Male)	109 ± 6.7	99 ± 5.3	31.3 ± 1.7	49.6 ± 2.3	88.7 ± 3.9	0.3 ± 0.01
Control (Female)	102 ± 4.9	86 ± 3.9	33.1 ± 3.1	41 ± 3.5	78 ± 1.7	0.25 ± 0.02
MRD (Male)	116 ± 9.1	87.9 ± 3.4*	25.9 ± 2.1*	48.9 ± 1.9	85.1 ± 2.3	0.29 ± 0.02
MRD (Female)	108 ± 7.3	75.1 ± 4.2*	28 ± 1.3*	43 ± 1.7	80 ± 2.5	0.23 ± 0.01

*P<0.01, N=8, compared with the corresponding control.

Table 3: Effect of MRD on Blood Lipids, Glucose, Triglycerides and Bilirubin.

Parameter	U/litre Plasma		
Treatment (sex)	ALT	AST	Alkaline Phosphatase
Control (Male)	27.4 ± 1.3	65.0 ± 3.9	95.0 ± 1.6
Control (Female)	24.9 ± 0.9	55.0 ± 4.1	83.0 ± 0.9
MRD (Male)	21.1 ± 0.7*	51.5 ± 3.2*	79.8 ± 4.2*
MRD (Female)	21.5 ± 1.1*	44.5 ± 1.7*	68.9 ± 2.9*

P<0.05, N=8 compared with its respective control

Table 4: Effect of MRD on hepatic enzymes in rats.

Parameter	U/Litre (Plasma)			mg % (Plasma)	µmole/g liver tissue
Treatment	ALT	AST	Alkaline Phosphatase	Bilirubin	Malon-aldehyde
Control (paraffin oil)	29.3 ± 0.9	70.0 ± 3.8	90.0 ± 6.1	0.3 ± 0.1	1.1 ± 0.09
CCl_4	91.7 ± 3.1	190.0 ± 6.9	261.0 ± 7.3	0.8 ± 0.2	3.7 ± 0.2
MRD	52.1 ± 2.9*	133.2 ± 11.3*	188.0 ± 4.9*	0.58 ± 0.1*	1.6 ± 0.15*

*P<0.01, N=8 compared with CCl_4-treated animals.

Table 5: Effect of MRD on carbon tetrachloride-induced hepatotoxicity in male rats.

vehicle for 13 weeks induced significant decreases in ALT, AST and alkaline phosphatase enzymes (P<0.05, N=8). Table 4 depicts the cumulative results.

Effect on blood ions

The blood levels of Na^+, K^+, Ca^{++} and Mg^{++} in the control male rats were 142 ± 0.9, 4.8 ± 0.03, 2.45 ± 0.03 and 0.9 ± 0.1 mmole/litre, respectively. The corresponding values in female rats were 133 ± 1.9, 4.6 ± 0.1, 2.3 ± 0.1 and 0.8 ± 0.07 mmole/litre. Treatment with MRD did not induce any significant changes in these levels.

Effect on urine production and the urinary concentrations of Na^+, K^+ and Ca^{++} ions

Treatment of both sexes of rats with MRD significantly increased the volume of urine voided from 3.95 ± 0.07 ml (male control) to 5.1 ± 0.1 ml and from 3.36 ± 0.04 ml (female control) to 4.51 ± 0.08 ml (P<0.05, N=8). The treatment increased the excretion of Na^+ from 0.128 ± 0.01 (control male) to 0.139 ± 0.02 mmole/litre and from 0.114 ± 0.03 (control female) to 0.123 ± 0.04 mmole/litre. These were insignificant increases (P>0.05, N=8). There were no significant changes in both K^+ and Ca^{++} excreted in urine. The treatment also decreased the urinary pH from 8.0 ± 0.3 (control male) and 8.1 ± 0.4 (control female) to 7.0 ± 0.5 and 6.9 ± 0.3, respectively. These changes were not significant (P>0.05, N=8).

Effect on carbon tetrachloride-induced hepatic damage in male rats

Treatment of male Wistar rats with a mixture of CCl_4 and paraffin (50:50) in a dose of 1 ml/kg (i.p.) induced severe damage to the liver on the third day following treatment of the animals (Table 5) as revealed by the significant increases in the hepatic enzymes ALT, AST, alkaline Phosphatase and in the hepatic level of malonaldehyde and the plasma level of bilirubin (P<0.01, N=8). MRD treatment significantly protected the animals against these increases (P<0.01, N=8).

Discussion

The results of this study clearly demonstrated the functional activity of *Myrica rubra* fruit juice that is formulated in form of a beverage drink. One of the first observed effects is its stimulant effect on the RBCs and their content of haemoglobin. Such actions were not followed in detail in this study. They may be due to stimulation of erythropoietin. The second observed clear effect is the ability of the drink to decrease both of the total cholesterol and the LDL. The metabolism of these lipids is generally regulated by a family of membrane-bound transcription factors called sterol regulatory element binding proteins, e.g., SREBP-1 [25] and SREBP-2 is reported to regulate the genes involved in cholesterol synthesis [26]. Furthermore, peroxisome prolifertor activated receptors, e.g., PPAR-alpha are involved in the lipids metabolism [27].

MRD is known to contain high concentration of proanthocyanidins [1]. These are condensed tannin polymers composed of various flavan-3-ol catechin units. The most available are the reddish procyanidins [2]. MRD constituents may act to suppress lipid metabolism regulatory proteins. Indeed, in a recent study, [7] revealed the ability of oligomeric proanthocyanidins to suppress SPEBP-2 [7] and to increase PPAR-α expression. Another possibility is that the proanthocyanidins and the various poly phenols present in MRD [1,2] may act to suppress fat absorption in the intestine. Previous studies revealed the inherent ability of proanthocyanidins to decrease hyperlipidemia in mouse model type 2 diabetes [9] and in streptozotocin-induced type 1 diabetes in rats [7]. The observed ability of MRD to decrease the levels of the hepatic enzymes during the subchronic treatment may be related to the ability of its proanthocyanidins to elevate the level of the Hepatoprotective glutathione. Indeed, these substances have been shown to elevate the level of glutathione in diabetic rats and mice.

Part of this study revealed the potential of MRD to act as a hepatoprotective against CCl_4-induced elevations in the plasma levels of ALT, AST, alkaline Phosphatase, bilirubin and malonaldehyde. The hepatotoxicity of CCl_4 is very well studied and is believed to occur as a result of generation of free radicals within the liver [28]. The initial step in this hepatotoxicity is the production of the trichloromethyl radical (-•CCl_3) via the enzyme cytochrome P450 subtypes (2E1, 2B1, 2B2 and 3A) [29,30]. The latter radical then interacts with molecular oxygen resulting in the production of the trichloromethyl peroxyl radical (-•O-O-CCl_3) [30]. This radical then initiates the peroxidation of the membrane phospholipids and the unsaturated fatty acids. All these peroxides act to damage the cellular components (e.g. mitochondria, endoplasmic reticulum and plasma membranes) with the resultant hepatotoxicity [28,30,31]. In addition, CCl_4 is reported to release various destructive cytokines such as TNFα [32]. Thus, the observed hepatoprotective effect of MRD may be highly related to the ability of some of its constituents to act as free radical scavengers. Indeed such antioxidant action has been observed for its constituents polyphenols, flavonoids and proanthocyanidins [6,7,33,34].

On a broad basis, the results of this study point clearly to the property of *Myrica rubra* fruit drink as an anti-anemic, a hypocholesterolemic and a hepatoprotective pointing to its potential as a new functional food.

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