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Antioxidant and Weight Loss Effects of Pomegranate Molasses

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ABSTRACT

Pomegranate molasses (PM) is a large compound of the eastern diets, yet no research has been performed on this product. In this study, we measured the total polyphenols content of PM, compared to fresh pomegranate juice, obtaining 252.28 and 79.49 mg of Gallic Acid equivalent/L respectively. The antioxidant effect of PM and juice was then measured in vitro using electrolysis as a free radical generating system. At the concentrations of 100 to 600 μ l PM has strong antioxidant properties (4 times more active than juice). Moreover, molasses or juice were added to the drinking water of mice (4 ml/l) during 11 weeks leading to a significant decrease of weight curve compared to control animals; also triglycerides and lipid peroxidation were decreased in the heart, lungs, and the liver, while superoxide dismutase activity increased. In conclusion, Pomegranate molasses possesses a powerful antioxidant activity and a weight loss effect in mice.

Keywords: Antioxidants, free radicals, pomegranate, weight loss.

INTRODUCTION

Several disorders such as cardiovascular diseases, diabetes, metabolic syndrome, cancer... have become more prevalent. Free radicals such as reactive oxygen species (ROS) have been implicated in their etiology. However, the defense mechanism of the body against ROS could be overwhelmed in many circumstances by an over-production of these species. Therefore, synthetic antioxidants and foods rich in antioxidants have been shown to boost our defense against ROS (Brambilla *et al.*, 2008). The Middle Eastern diets contain many foods, among which the pomegranate molasses, are believed to have antioxidant effects but without much scientific evidence. This substance is an extract from pomegranate (*punica granatum L.*). This species grows generally in Asia, the Mediterranean border, and the American continent. Pomegranate is considered one of the oldest fruits and one of the earliest to appear in human diet. The pericarp, the inner lamella, and the part consumed constitute 38, 10 and 52% respectively of the total weight. The juice is 78% of the portion used (45-61% of total weight) and the seeds are 22% (El-Nemr *et al.*, 1990). Pomegranates are usually consumed fresh; however they are also a source of juice and molasses. The juice contains a considerable amount of carbohydrates, ascorbic acid, Vit B, pectin, cellulose, tannins, and ash. Moreover, the pericarp is a good source of polyphenols such as anthocyanin, leucoanthocyanins, catechins, and flavonoides and contains about 30 % of tannins.

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This could explain the benefit of pomegranate compounds when used as additives or anti-bacterial due to phenolics, pigments and citric acid. Because of their rich content in tannins, the pericarp, the roots, and the peel are used in medicine against dysentery as they are included in the pharmaceutical preparations containing biologically active substances (El-Nemr *et al.*, 1990; Schubert *et al.*, 1999).

Negi *et al.* (2003) reported that pomegranate peel extracts exert anti-oxidant and anti-mutant activities in vitro due to their content of polyphenols (tannins, ellagic and gallic acids). These substances have been used in the preparation of cosmetics and tinctures as well as in therapeutic formulas and food recipes. On the other hand, Gil *et al.* (2000), Aviram *et al.* (2000), Singh *et al.* (2002) have reported an anti-oxidant and anti-sclerotic effects of pomegranate syrup on animal models in vitro. Furthermore, human studies have shown that daily consumption of pomegranate juice lowers blood pressure in hypertensive subjects, delays the atherosclerotic process and increases the total antioxidant status of blood. Pomegranate juice has a remarkable ability to decrease oxidative stress by 40–80% and to increase the antioxidant enzymes-paaronases (serum PON1 and macrophage PON2) by 50–100% (Aviram *et al.*, 1998, 2004). Moreover, cholesterol homeostasis is improved by a decrease in LDL total cholesterol levels especially in diabetics (Jurenka, 2008).

Pomegranate molasses, a concentrated pomegranate juice and widely consumed in the Middle East, may be rich in more efficient antioxidants than that found in the juice. No research has been done on this substance so far. The objective of our work is to fill this gap and determine: 1) the total polyphenols which are known for their strong antioxidant properties in juice and pomegranate molasses 2) the anti-oxidant activity of the two substances in vitro in the presence of ROS generated by electrolysis, 3) the weight curve and lipid profile in rats given pomegranate juice or molasses in their beverage. 4) Histological studies of the heart, liver, and lungs submitted to free radical generation done by electrolysis.

MATERIALS AND METHODS

We used fresh pomegranate juice (PJ) and molasses (PM) made from a bitter pomegranate variety grown in Lebanon. The production of molasses consisted of peeling the fruits, dispersing the grains and pressing them manually to have a juice. The juice is boiled for more than six hours in order to obtain a concentrated substance called “molasses.”

Measurement of polyphenols

Total phenolic contents of pomegranate juice and molasses were measured, according to Singelton *et al.* (1999) using Folin-Ciocalteu reagent and gallic acid as standard. Briefly, 0.1 ml of the juice or molasses was taken in a volumetric flask, 46 ml of distilled water and 1 ml of Folin-Ciocalteu reagents were added and the flask was shaken thoroughly. After 3 min, 3 ml of 2% Na_2CO_3 solution was added and the mixture was allowed to stand for 2 h with intermittent shaking. Absorbance was measured at 760

nm. The same procedure was repeated to all standard gallic acid solutions (0–1000 mg, 0.1 ml^{-1}), a standard curve was obtained and the results expressed as mg GA equivalent/L.

Generation of free radicals by electrolysis

The physiological solution is first prepared constituting of: NaCl (137 Mm), KCl (2.7 mM), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (1 mM), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (1.5 mM), $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (0.4 mM), and NaHCO_3 (12 mM). The electrolysis apparatus is made up of a stimulator adjusted at 10 mA, by a sensitive multimeter, and of wires connecting it to two platinum electrodes which are in turn introduced into a bath containing 20 ml of the prepared solution, and a magnetic stirrer to speed up mixing and homogenizing the medium (Chahine *et al.*, 1991, Lecours *et al.*, 1998). Every one minute during electrolysis, 1 ml is taken from the physiological solution and added to 2 ml of N, N - diethyl-P-phenylendiamine (DPD) (25 mg/ml) in a specific tube, vortexed and measured using spectrophotometer at 515 nm (control). Similarly, the electrolysis of the physiological solution is performed in the presence of 200 μl , 400 μl and 600 μl of pomegranate juice or of molasses. The DPD solution gives a pink color proportional to the free radicals generated.

Pomegranate juice and molasses in drinking water of mice

Animal protocols were conducted in accordance with the international guideline for animal care and approved by the Central Research Committee, Lebanese University. Our experiments were done on adult male albino mice (*Mus Musculus*) weighing 22 ± 4 g. The animals were housed under standard laboratory conditions and were fed with commercial rodent feed and tap water ad libitum for one week in the new environment ($24.0 \pm 0^\circ\text{C}$ temperature & 55–65% relative humidity and 12 hours light/dark cycle) before experiments were carried out. Any animal showing abnormal behavior was excluded from the study. The animals were then randomly divided into 3 groups of 6 animals each, placed into cages.

- The first group was given regular water (control).
- The second group was given water with pomegranate molasses at a concentration of 4 ml/l.
- The third group was given water with pomegranate juice at a concentration of 4 ml/l.

The animals' food and water intake was observed closely along with weekly measurements of their weight. At the end of the eleventh week, the animals were anesthetized and dissected and unusual organ abnormalities were noticed. Then, the liver, lungs, and the heart were excised and weighed, and then in turn subjected to biochemical and histological analysis.

Biochemical assays

Fragments of 0.08 g were taken from the three organs and completely cleaned from blood. Each fragment was homogenized for 2 min on ice by the potter in 10 ml of phosphate buffer solution and then stored at -80°C till assay.

Triglycerides

This assay was performed at the Baabda University Hospital (Lebanon) using standard kits for plasma adapted to tissue homogenate (Mateescu *et al.*, 1995).

Lipid hydroperoxide assay

Lipid peroxidation (LP) levels were determined using a lipid hydroperoxide assay kit which measures the redox reactions with ferrous ions (Cayman Chemical Co USA). Because hydroperoxides are unstable they reacted with ferrous ion to produce ferric ions. The resulting ions are detected using thiocyanate ion as the chromogen. To avoid over-estimation of lipid hydroperoxides, they were extracted from samples by chloroform; then the absorbance of each tube is measured at 500 nm.

Superoxide dismutase assay

Superoxide dismutase (SOD) levels were determined using a SOD assay kit containing tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine (Cayman Chemical Co USA). Briefly, one unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of radical superoxide. This assay measures the three types of SOD (Cu / Zn, Mn and FeSOD). A plate was incubated on the shaker for 20 min at room temperature and the absorbance measured at 440-460 nm using a plate reader.

Histological observation

The remaining part of the three organs was introduced in the bath containing 20 ml of physiological solution and subjected to five-minute electrolysis, then placed in a 20% formalin solution for histological studies. They were fixed on paraffin and counterstained with hematoxylin-eosin, then sliced with microtome for light microscopy.

Statistical Analysis

The data are expressed as mean \pm SD. Statistical comparisons were performed by One-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test. The results were considered statistically significant if the p value was less than 0.05.

RESULTS

Dosage of total polyphenols

Table 1, show the mean levels of total polyphenols found in PM and PJ expressed in mg GA equivalent/L.

Table. 1: Analysis of total polyphenols in six different samples of pomegranate molasses and juice. (GA: Gallic acid)

	Total Polyphenols (mg GAequivalent/L)
Molasses	252.28 \pm 33.67
Juice	79.49 \pm 25.25

Electrolysis

Figure 1 illustrates the absorbance of the physiological solution after five-minute electrolysis. The absorbance and hence

the production of ROS are strongly diminished in the presence of molasses but less in presence of juice. The effect of molasses or juice is dose-dependent of the concentration used. Thus, the strong antioxidant substances found in molasses are acting as ROS scavengers.

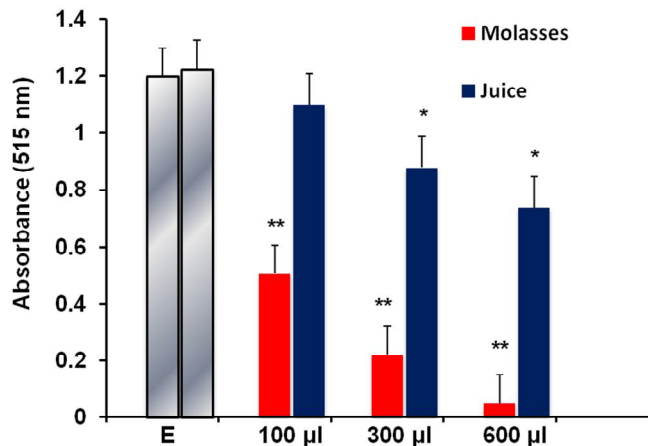


Fig. 1: Effects of pomegranate juice or molasses added to the physiological solution after 5 min of electrolysis. E =control (physiological solution alone). *P < 0.05 ** p<0.01, versus control.

Weight curve

Figure 2 show that mice in the control group had a normal weight accumulation. Weight gain in the group that received the pomegranate molasses and juice was significantly lower than that of the control group. However the group that received the juice had a lower accumulation of weight which later started to increase after 8 weeks. In the group that received the molasses, a slight loss of weight is immediately observed followed by a slow regain of weight after 8 weeks; the weight remained stable near baseline values.

Biochemical study

Table 2 shows that TG and LP tissue levels were significantly decreased in the 3 organs of the mice that received the molasses. These levels were slightly diminished in the group that received the juice in comparison to the control group. On the other hand, SOD activity increased significantly in the group of mice given molasses, but slightly in the group given juice.

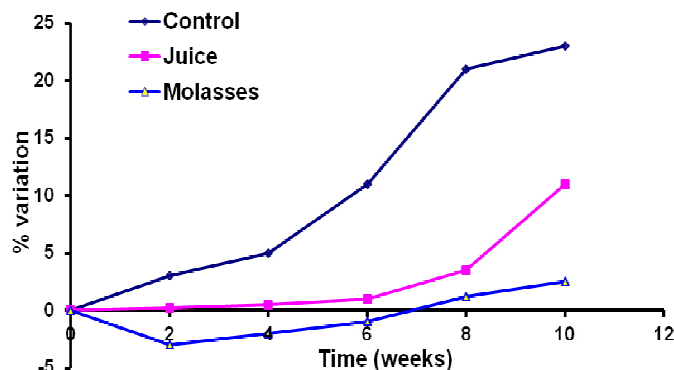


Fig. 2: Weight curve of mice that received pomegranate molasses or juice (4 ml/l) added to their drinking water. Results are expressed as percentage of initial weight.

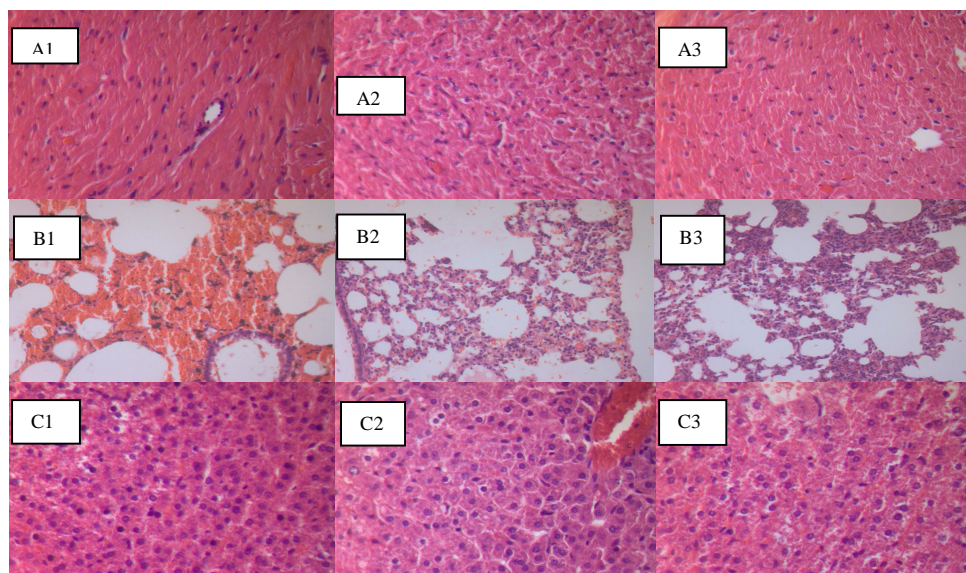


Fig. 3 : Micrographs of the studied organs (original magnification $\times 400$): A1-Control heart, A2-Electrolyzed heart, A3 -Electrolyzed heart + PM; B1-Control lung, B2-Electrolyzed lung, B3- Electrolyzed lung + PM; C1-Control liver, C2-Electrolyzed liver, C3- Electrolyzed liver + PM

Histological study

Examination of the organs of treated animals shows lighter color than the control group, and the fat around the organs had almost disappeared. Histopathological studies on the isolated organs (heart, lung, and liver) submitted to 5 min electrolysis, resulted in structural damage as compared to control group. Concerning the heart and liver, there was hyalinization, and a loss of striations, whereas extracellular spaces became larger (Figure 3: A2). Extracellular spaces in the lungs also became larger (Figure 3: B2, C2); while in animals taking pomegranate molasses, a protection is provided to these organs (Figure 3: A3, B3, C3). These junctions were of medium size in the group that received the juice (slides not shown).

DISCUSSION

Obesity is increasing worldwide. This is believed to be related to the excessive consumption of food rich in calories as well as bad eating habits (binge eating, night eating, high fructose corn syrup, alcohol abuse and smoking etc.). However, good eating

habits, along with the ingestion of food rich in antioxidants, may play a protective role against many diseases. It is known that equilibrium between oxidants and antioxidants is crucial to the body, but the virtues of anti-oxidant substances have been amplified in the paramedical literature without any solid scientific basis. Consequently, it would be important to look into the products consumed by certain population that have a protective effect on the organism, and encourage other population to consume it for its beneficial effect against certain diseases such as the metabolic syndrome.

Polyphenols are the most abundant antioxidants in most diets. Their intake is 10 times higher than the intake of vitamin C and 100 times higher than that of vitamin E or carotenoids. As reviewed by Middleton *et al.* (2000), polyphenols exert antioxidant activities. Polyphenols like catechin or quercetin can directly scavenge ROS, such as superoxide radical, hydrogen peroxide, or hypochlorous acid, which can be very deleterious by damaging lipids, proteins and DNA (Pannala *et al.*, 1997 ; Binsack *et al.*, 2001). The phenolic core can act as a buffer and capture electrons from ROS to render them less reactive. Furthermore, polyphenols and quercetin in particular, can chelate metals like iron involved in free radical formation (Korkina and Afanas'ev, 1997 ; Nijveldt *et al.*, 2001). Indirectly, polyphenols can interfere with the cellular detoxification systems, such as superoxide dismutases (SOD), catalase or glutathione peroxidases (Krinsky, 1992; Herber *et al.*, 2007). Besides, polyphenols can inhibit enzymes generating ROS as xanthine oxidase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Jurenka, 2008).

Pomegranate is among the richest fruit in polyphenols. It is logical to hypothesize that fresh pomegranate juice should have a more considerable amount of polyphenols than that found in molasses obtained after 6 hours of heating. One can consider that high temperature should deteriorate polyphenols in PM as severe thermal treatment can alter the bioavailability of polyphenols by

Table. 2: Triglycerides levels (TG), lipid peroxidation (LP) and superoxide dismutase (SOD) levels in different organs of both mice groups, the received and non-received pomegranate juice (PJ) or molasses (PM) in drinking water versus control (C). $P < 0.05$ versus control, $n = 6$

	TG (mg/dL)	LP (nmol/mg)	SOD (U/100mg)
Liver			
C	19.3 \pm 3.0	127.5 \pm 13.5	472.8 \pm 41.7
PJ	15.2 \pm 2.8*	107.8 \pm 10.7*	492.2 \pm 42.0
PM	6.4 \pm 2.5 *	82.9 \pm 10.8*	544.0 \pm 35.6*
Lungs			
C	10.1 \pm 2.2	65.4 \pm 5.6	78.8 \pm 6.9
PJ	6.7 \pm 1.9	60.0 \pm 4.8	86.5 \pm 7.9
PM	4.5 \pm 1.2*	53.9 \pm 3.4*	101.3 \pm 8.6*
Heart			
C	14.0 \pm 2.6	79.5 \pm 6.1	113.6 \pm 10.6
PJ	11.8 \pm 2.5	70.3 \pm 5.4	130.2 \pm 14.5
PM	5.4 \pm 1.8 *	67.7 \pm 5.9*	145.3 \pm 17.8 *

decreasing the absorption of active compounds, due to the formation of oxidized products (Porrini and Riso, 2008; Visioli *et al.*, 2011). On the contrary, according to our results polyphenols in molasses are four times greater than those found in the juice. Moreover, at a very low concentration (100 to 600 µl), PM has the strongest antioxidant properties *in vitro* compared to PJ. This could indicate that high temperature does not alter the antioxidant activity of PM against ROS. On the other hand it seems that the high temperature helps polyphenols to be released from pomegranate fruit cell as there is no extraction with a solvent in the preparation of pomegranate molasses.

In relation with our results, extracts from the whole fruit have stronger antioxidant properties than those of the juice. This difference in the activity is probably due to the presence of tannins coming found in the pericarp of the fruit. This explanation was proposed by Gil *et al.* (2000) who studied the relationship between the different techniques of extraction and the polyphenolic contents in the pomegranate juice. The authors showed that the antioxidant properties are due to the polyphenolic content. They also confirmed, by using the Folin-Ciocalteu method, that commercial pomegranate juice contains higher concentration of polyphenols than that produced manually.

We conducted a pilot study on mice by giving them pomegranate juice and molasses, two byproducts widely used by Lebanese consumers, in order to test their efficacy. Despite the low number in our animal group, the results obtained under our experimental conditions showed that PJ, and to a greater extent PM induced weight loss in the animals. These findings were corroborated by a decrease in the TG levels and lipid peroxidation; while SOD activity is increased in the heart, lungs, and liver. At the same time the protective effects of the PM against ROS generated by the electrolysis were histologically demonstrated.

Different studies have been done on PJ and on other pomegranate extracts. Hence, an *in vitro* assay using four separate testing methods have demonstrated that pomegranate juice and seed extracts have 2-3 times more antioxidant capacity than that found in red wine or green tea (Gil *et al.* 2000). Studies in rats and mice have confirmed that the antioxidant properties of a pomegranate by-product extract made from whole fruit without the juice, and also showed a 19 % reduction in oxidative stress in mouse peritoneal macrophages, a 42 % decrease in cellular lipid peroxide content, and a 53 % increase in reduced glutathione levels (Rosenblat *et al.* 2006). Another study demonstrated that CCl₄ caused liver damage in rats, whereas pretreatment with a pomegranate peel extract enhanced or maintained the free-radical scavenging activity of the hepatic enzymes catalase, SOD, and peroxidase, and resulted in 54 % reduction of lipid peroxidation values compared to controls (Chidambara *et al.*, 2002). Negi *et al.* (2002) reported that these extracts exert antioxidant and anti-mutagenic activities *in vitro* due to the polyphenols such as tannins, Ellagic and Gallic acids used in the tincture preparations, cosmetics and in food recipes (Nasr *et al.*, 1996). In addition to this, Gil (2000), Aviram (2000), Singh (2001, 2002) and a study by

Negi (2003) described the antioxidant and anti-sclerotic activities of pomegranate juice on animal and human models *in vitro*.

Pomegranate and its constituents have safely been consumed for centuries without any side effects. Studies of pomegranate constituents in animals at concentrations and levels, commonly used in folk and traditional medicine, note no toxic effects (Vidal *et al.*, 2003). Toxicity of the polyphenol antioxidant punicalagin, abundant in pomegranate juice, was tested in rats. No toxic effects or significant differences were observed in the treated group compared to controls, which was confirmed via histopathological analysis of rat organs (Cerdeira *et al.*, 2003).

CONCLUSION

In conclusion, PJ and PM present constantly in the beverage of mice at low doses are able to resist weight gain, induce the loss of fat stores, decrease tissue necrosis, and protect against deleterious effects of ROS. Nevertheless the loss of fat stores may render the animals hypothermic; the results obtained *in vivo* could lead to the development of pharmaceutical or natural product that reduces fat mass, if investigated in clinical studies. Any how we are just started the chemical analysis of the PM in order to better understand its mechanism of action.

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