

## Antioxidant and eicosanoid enzyme inhibition properties of pomegranate seed oil and fermented juice flavonoids

Shay Yehoshua Schubert<sup>a</sup>, Ephraim Philip Lansky<sup>b</sup>, Ishak Neeman<sup>a,\*</sup>

<sup>a</sup> Laboratories of Food Engineering and Biotechnology, Technion—Israel Institute of Technology, Haifa 32000, Israel

<sup>b</sup> Rimoni Corporation, Science Park, Nesher, Israel

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### Abstract

The antioxidant and eicosanoid enzyme inhibition properties of pomegranate (*Punica granatum*) fermented juice and seed oil flavonoids were studied. The pomegranate fermented juice (pfj) and cold pressed seed oil (pcpso) showed strong antioxidant activity close to that of butylated hydroxyanisole (BHA) and green tea (*Thea sinensis*), and significantly greater than that of red wine (*Vitis vitifera*). Flavonoids extracted from pcpso showed 31–44% inhibition of sheep cyclooxygenase and 69–81% inhibition of soybean lipoyxygenase. Flavonoids extracted from pfj showed 21–30% inhibition of soybean lipoyxygenase though no significant inhibition of sheep cyclooxygenase. The pcpso was analyzed for its polyphenol content and fatty acid composition. Total polyphenols in pcpso showed a concentration by weight of approximately 0.015%. Pcpso fatty acid composition showed puniic acid (65.3%) along with palmitic acid (4.8%), stearic acid (2.3%), oleic acid (6.3%), linoleic acid (6.6%) and three unidentified peaks from which two (14.2%) are probably isomers of puniic acid (El-Shaarawy, M.I., Nahpetian, A., 1983). Studies on pomegranate seed oil. *Fette Seifen Anstrichmittel* 83(3), 123–126). © 1999 Elsevier Science Ireland Ltd. All rights reserved.

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### 1. Introduction

Pomegranate (*Punica granatum*), a small tree originating in the Orient, belongs to the Punicaceae family (Harde et al., 1970). Pomegranate is grown mainly in Iran, India and the USA, but also in most Near and Far East countries. The

main use of pomegranate is as table fruit, but large amounts are used in the beverage and liquor industries (Nagy et al., 1990). The pericarp, containing up to 30% tannins, is used in tanning leather (Duke and Ayensu, 1985).

In folk medicine, pomegranate preparations, especially of the dried pericarp, but also of the roots, barks of the tree and roots, and the juice of the fruit, are employed as per orum medication in

\* Corresponding author. Fax: +972-4-832-0742.

the treatment of colic, colitis, diarrhea, dysentery, leucorrhoea, menorrhagia, oxyuriasis, paralysis and rectocele, and as external applications to caked breast (Duke and Ayensu, 1985) and to the nape of the neck in mumps (Boulos, 1983) and headache (Ayensu, 1981). Further, a number of therapeutic actions of these materials have been described including vermifugal, taenicial, astringent, antispasmodic, antihysterical, diuretic, carminative, sudorific, galactagogue and emmenagogue (Bianchini and Corbetta, 1979).

Flavonoids, a broad class of polyphenolic compounds widely distributed among photosynthesizing cells, possess an impressive array of pharmacological activity (Hasten, 1983). These include: free radical scavenging, inhibition of a vast spectrum of enzymes, and estrogenic activity. Consequently, a potential role for these compounds in several therapeutic functions is apparent. As anti-inflammatory agents, flavonoids may be effective against parodontitis and local pain, without the gastric irritating effects of aspirin and other non-steroidal anti-inflammatory drugs (which also act through inhibition of cyclooxygenase-catalyzed prostaglandin formation). Flavonoids have also been suggested as cancer-protective agents, if not therapeutic ones (Hasten, 1983) and the consumption of dietary flavonoids was inversely correlated with coronary heart disease in a population of elderly men (Hertog et al., 1993). In the present work we studied pcpso and pfj for their antioxidant activity (Hammerschmidt and Pratt, 1978) and inhibitory effects on lipoxygenase and cyclooxygenase, key enzymes in the eicosanoids pathway. Lipoxygenase inhibition was determined using soybean 5-lipoxygenase (Grossman and Zakut, 1979) and cyclooxygenase inhibition using sheep cyclooxygenase from sheep vesicular glands (Van der Ouderaa et al., 1977).

## 2. Materials and methods

### 2.1. Plant material

Plant material was collected by one of the authors (E. Lansky) through the courtesy of the late Professor Dan Palevitch from the cultivar

collection from the Neve Yaar Research Station, Volcani Agricultural Research Organization, Ministry of Agriculture, State of Israel in the southern Galilee. A sample of mixed cultivars was employed.

### 2.2. Preparation of fermented plant juice (pfj)

The seeds of the fruit containing the intact juice sacs were manually separated from the pericarps, and the sacs ruptured by very light agitation in an electric blender for 2–3 s. The mixture of the juice and the seeds was then added to a high quality sterilized plastic jug (ordinarily used for storing spring water). To 16 l of this mixture was added 5 g of wine yeast, *Saccharomyces bayanus* (Lalvin EC-1118) obtained from Lallemand, Montreal, Canada. A sterile surgical glove was affixed to the neck of the bottle with a rubber band which served as a pressure release valve, and fermentation was allowed to proceed at room temperature until complete (10 days). A portion of the wine was then decanted and gradually evaporated to one-tenth of its original volume to yield the pfj extract used in the study.

### 2.3. Preparation of cold pressed pomegranate seed oil (pcpso)

After the completion of fermentation of the juice, the seeds were removed by straining and dried in the sun, or alternatively, over an electric radiator. The dried seeds were then cold pressed in a Tiby Press Type 55 machine with a 7-mm nozzle manufactured by Skeppsta Maskin of Orebro, Sweden. A 5.3% yield of oil per dry weight of seeds was obtained.

### 2.4. Flavonoid extraction from pcpso

Flavonoid extraction from the pcpso was accomplished with the method previously described for olive oil (Vazues et al., 1973). A 10-g aliquot of pcpso was moved with 50 ml hexane in a separation funnel and polyphenols extracted with three volumes of 60% methanol. The methanol phase was then moved to a second separation funnel and washed with 20 ml hexane. The

methanol phase was then collected and dried with anhydrous  $\text{Na}_2\text{SO}_4$  and again dried in a vacuum evaporator at  $40^\circ\text{C}$ . The resultant polyphenols were resuspended in methanol and extracted with three portions of chloroform, each half the volume of the methanol phase. The chloroform was removed and the methanol dried again in the vacuum evaporator at  $40^\circ\text{C}$ . The polyphenols were resuspended in water and extracted with petrol ether (60–80) until a clear organic phase was obtained. The water phase was saturated with  $\text{NaCl}$  and extracted with four portions of ethyl acetate (EA), each a third of the water phase volume. The EA fractions were collected and dried with anhydrous  $\text{Na}_2\text{SO}_4$ . The EA was dried in a vacuum evaporator and the polyphenols resuspended in methanol and kept at  $-20^\circ\text{C}$ .

### 2.5. Flavonoid extraction from *pif*

The pomegranate fermented juice extract was combined with two times its volume of EA, shaken vigorously, and left for 8 h. The EA phase was then dried in the vacuum evaporator at  $40^\circ\text{C}$ , and polyphenols resuspended in methanol.

### 2.6. Determination of antioxidant activity

Antioxidant activity was determined by measuring the coupled oxidation of carotene and linoleic acid (Fluka, Germany), a modification of a method previously reported (Hammer-schmidt and Pratt, 1978). Approximately 10 mg *trans*- $\beta$ -carotene (type 1 synthetic, Sigma, St Louis, MO) was dissolved in 10 ml of chloroform. The carotene–chloroform solution, 0.2 ml, was pipetted into a boiling flask containing 20  $\mu\text{l}$  linoleic acid and 200  $\mu\text{l}$  Tween-40 (Sigma). After removal of the chloroform with  $\text{N}_2$ , 50 ml of double distilled water (DDW) was added to the flask with vigorous swirling. To tubes containing the putative antioxidants in 2 ml ethanol, 5 ml of the aliquots of these emulsions were each added to final concentrations by weight of 0.01%. Spectrophotometric readings at 470 nm (Ultraspec II spectrophotometer) were

taken immediately after addition of the emulsion to the antioxidant solution against a blank containing absolute ethyl alcohol (Carlo Erba, Italy). The tubes were stoppered and placed in a water bath at  $50^\circ\text{C}$ , with readings taken at 15-min intervals for 90 min. Controls consisted of butylated hydroxyanisole (BHA, Sigma), green tea (Bi Luo Chun, Hua Sheng Wen Ju Factory, Su Zhou, China) and red wine (Cabernet Sauvignon, Barkan Winery, Israel, 1995).

### 2.7. Polyphenol determination

Polyphenols were determined using a spectrophotometric method (AOAC, 1990). Folin Danis reagent ( $\text{Na}_2\text{WO}_4 \cdot 2 \text{H}_2\text{O}$  100 g) and phosphomolybdic acid (20 mg, 50 ml) were distilled for 2 h in reflux, chilled and diluted to 1 liter in DDW. Subsequently, 35 g  $\text{Na}_2\text{CO}_3$  was dissolved in 100 ml DDW, left overnight for crystallization and filtered.

To obtain a calibration curve, to different concentrations of tannic acid were added 0.5 ml Folin Danis, then 1 ml  $\text{Na}_2\text{CO}_3$  solution, followed by DDW until a total volume of 10 ml was achieved. Readings were taken at 760 nm after 30 min. Polyphenols were determined in a similar manner, but instead of tannic acid the flavonoid sample was used.

### 2.8. Cyclooxygenase preparation

Cyclooxygenase was obtained from sheep vesicula seminalis (Yamamoto, 1982). Ten vesicles from freshly slaughtered sheep were homogenized in three volumes of potassium phosphate buffer, 50 mM, pH, 7.4, containing 1 mM EDTA (Fluka Germany). The homogenate was centrifuged at  $12\,000 \times g$  for 15 min and the surfactant centrifuged at  $100\,000 \times g$  for 1 h. The pellet containing the microsomal fraction was dissolved in Tris–HCl buffer (Sigma) containing 1% Tween 20 (Sigma), 0.1 mM EDTA and 20% glycerol, centrifuged at  $27\,000 \times g$  for 30 min, and the surfactant containing the dissolved enzyme was collected into small containers and kept at  $-70^\circ\text{C}$ .

### 2.9. Determination of the activity of cyclooxygenase

The activity of cyclooxygenase was determined using a polarographic assay employing an O<sub>2</sub> electrode. Oxygen uptake was measured as the change in dissolved oxygen concentration catalyzed by cyclooxygenase and measured using a Clark (O<sub>2</sub>) electrode. The substrate was arachidonic acid 90% purity (Sigma), 0.1 mM in Tris–HCl, pH 8.0 buffer and Hemin (chlorid) (Fluka Germany) 1 M. The enzyme was preincubated for 2 min with the inhibitor, then added to the reaction cell containing the substrate at 30°C. Hydroquinone (Fluka Germany), 0.041 mg/ml, was added immediately prior to the reaction. Indomethacin (Sigma), a known cyclooxygenase inhibitor, was used as a positive control.

### 2.10. Determination of the activity of lipoxygenase

The activity of soybean lipoxygenase (Sigma) was similarly determined using a polarographic, oxygen-measuring assay. Oxygen uptake was assessed as the change in dissolved oxygen concentration catalyzed by lipoxygenase and measured using the aforementioned Clark electrode. The substrate in this case was linoleic acid, 7.5 mM, dispersed in water with the help of Tween 20, and diluted with 0.2 M sodium phosphate buffer to pH 6.5. The enzyme was preincubated for 2 min with each putative inhibitor and then added to the reaction cell containing the substrate at 30°C. Green tea, red wine and BHA were employed as positive controls.

### 2.11. Determination of pcpso fatty acids composition

Following extraction of polyphenols, 0.5 ml pcpso was refluxed for 2 h with 100 ml of 1% H<sub>2</sub>SO<sub>4</sub>. After cooling, the mixture was placed in a separation funnel and 300 ml H<sub>2</sub>O added. The oil was extracted with three volumes of 50 ml petrol ether 40–60 (Frutaroum Israel). The fatty acid methyl esters were then analyzed in an HP 5890 series II gas chromatograph equipped with a

flame ionization detector and coupled to a Kunirun computing integrator. Column used 6' # 0.25E # 2 mm Chromosorb W-HP 100/120 coated with 10% FFAP. Column temperature was programmed from 190 to 210°C. Nitrogen was the carrier gas. Mixtures of authentic standard fatty acids methyl esters were chromatographed under the same conditions for comparison.

## 3. Results and discussion

In Fig. 1, the antioxidant activities of pomegranate fermented juice (pjf) extract and pomegranate cold pressed seed oil extract (pcpso) are compared with the chemical antioxidant standard, BHA, and the most popular botanical antioxidants, green tea and red wine. As can be noted, the antioxidant activity of both pomegranate fractions was significantly superior to that of red wine. Conversely, the antioxidant activity of the pomegranate fractions approached, but did not surpass, the antioxidant activity of either a premium green tea or BHA.

The measurement of antioxidant activity depicted in the figure is accomplished through a coupled oxidation of linoleic acid to a variety of future oxidation-provoking oxidation products, and  $\beta$ -carotene, whose pigment is readily and quantifiably detectable with spectrophotometry. As the  $\beta$ -carotene loses its color, oxidation is proceeding, not only of the  $\beta$ -carotene itself, but

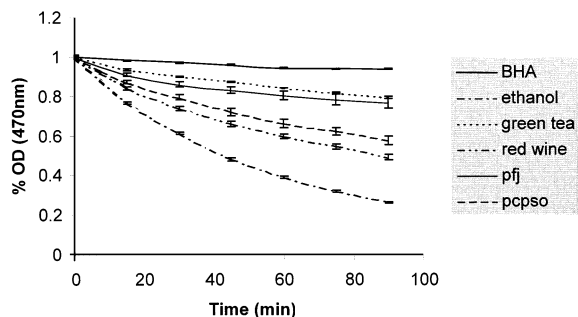


Fig. 1. Comparison of antioxidant activity of pjf extract and pcpso extract to BHA, green tea and red wine extracts. Antioxidant concentration, 0.01%. Negative control, ethanol ( $n = 3$ ).

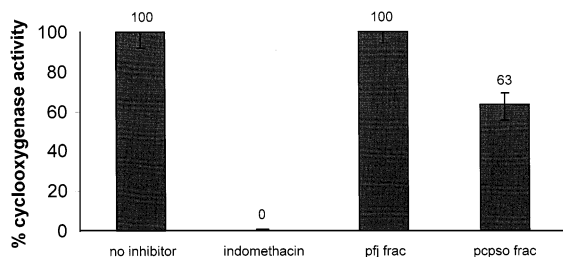


Fig. 2. Cyclooxygenase inhibition by pjf extract and pcpso extract. Inhibitor, 5  $\mu$ g. Positive control, indomethacin ( $n = 5$ ).

also of linoleic acid. Thus the more bleached out the solution, and the lower the values in the figure, the greater is presumed to be the oxidant activity. The values are an expression of the measurable optical density (*OD*) of the solution over time (*T*), i.e. *OD/T*.

Fig. 2 depicts the inhibition of the eicosanoids pathway enzyme cyclooxygenase, responsible for the 'cyclic' transformation of arachidonic acid to prostaglandins and thromboxane. The prostaglandins, so named because they were originally discovered in prostate glands, are key mediators of inflammation, which is why the so-called non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, acetyl-salicylic acid (ASA) and indomethacin are effective—because they inhibit cyclooxygenase. Prostaglandins, as well as thromboxane, are involved in clotting mechanisms, again why aspirin is used prophylactically to prevent thromboses. Here, the pomegranate fractions from both the pjf and pcpso are employed at a standard weight of 5  $\mu$ g total polyphenols, obtained as previously described. The height of the bar graphs is proportional to the degree of activity of cyclooxygenase, and inversely proportional to the degree of enzyme inhibition. As can be readily observed, the activity of this enzyme was eliminated with the NSAID, indomethacin. The pomegranate fermented juice fraction (pjf) failed to show any inhibition, but pomegranate cold pressed seed oil (pcpso) fraction effected 37% inhibition of cyclooxygenase (i.e. 63% of total cyclooxygenase activity).

In Fig. 3, the activity of the second major eicosanoid pathway enzyme, lipoxygenase, is expressed by the height of the bar graphs. The

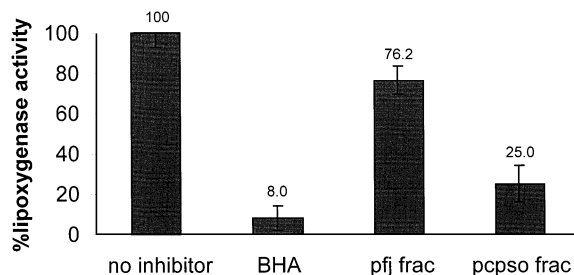


Fig. 3. Lipoxygenase inhibition by pjf extract and pcpso extract. Inhibitor, 5  $\mu$ g. Positive control, BHA ( $n = 5$ ).

industrial antioxidant BHA effected a 92% inhibition of this enzyme, the pomegranate fermented juice fraction (pjf) a 23.8% inhibition, and 75% inhibition by the pcpso fraction.

Lipoxygenase also catalyzes transformations of the starting substrate arachidonic acid, but in a parallel 'linear' rather than 'cyclic' pathway, to produce the leukotrienes (Johnson et al., 1983). Like prostaglandin and thromboxane, leukotrienes also play important, though as yet incompletely understood, roles in inflammation, atheromatous plaque formation and platelet aggregation, and also, apparently, asthma (Spector, 1995).

Table 1 reveals the result of quantitative analysis of the cold pressed pomegranate seed oil (pcpso) by gas chromatography (GC) and mass spectrometry (MS). A full 65.3% of this oil, in agreement with previous investigation (El-Shaarawy and Nahpetian, 1983), is shown to be punicic acid, a fatty acid which seems to be unique to pomegranate seed oil. The full meaning

Table 1  
Pcpso fatty acid composition<sup>a</sup>

Fatty acid	Percent of total oil
16:0 palmitic	4.8
18:0 stearic	2.3
18:1 oleic	6.3
18:2 linoleic	6.6
18:3 punicic	65.3
Unknown	0.4
Unknown	8.3
Unknown	6.0

<sup>a</sup> Analysis by gas chromatography/mass spectrophotometry.

and implications of this compound in human physiology, nutrition and medicine remains to be elucidated. Further, 14.3% of the mixture of fatty acids remained unidentifiable, even though both the GC and MS were run twice with the sample on separate occasions.

#### 4. Conclusion

This study clearly demonstrates a decided antioxidant activity of a pomegranate fermented juice and seed preparation and also of cold pressed pomegranate seed oil. Consequently, a role for these materials as potential natural food preservatives and/or health protective or therapeutic agents is suggested.

The enzyme inhibition properties of the fermented juice preparation and cold pressed pomegranate seed oil remain to be amplified in future investigations. Cold pressed pomegranate seed oil possesses uniqueness both in fatty acid composition and also range of estrogenic compounds including the isoflavonic phytoestrogens, another important phytoestrogen, coumestrol, and the steroidal estrogen estrone (Moneam et al., 1988), and to exert a potent estrogenic effect *in vivo* in two different animal models (Sharaf and Nigm, 1964). In this study, a potential role for pomegranate seed oil as a cardioprotective and also as an anti-inflammatory medicament for internal and/or external applications, is suggested.

The procedure for drying the seeds in the sun was less than ideal, and may have hindered, though most likely not potentiated, the antioxidant and enzyme inhibition properties of the oil. In the future, less potentially physiologically disruptive methods of drying should be explored.

The power of the fermented juice is less clear. In the present study, fermentation of the juice was undertaken both to conform to the parallel used in wine, the source of the so-called French paradox, whereby the cardioprotective effect of red wine in revelers of high fat foods has been attributed to its antioxidant activities (Ramathnam et al., 1995), and also as a means of effecting a gentle ethanolic/aqueous extraction of

the seeds. It should be recalled that the juice was fermented here with the seeds inside, and also aged for an additional few months, again with the seeds still contained with the juice. Hence, we are as yet unable to differentiate between the partial extraction of the seed oil into the fermented juice, and the actual antioxidant and enzyme inhibition properties of the juice itself, both in an unfermented and fermented state. In future studies, we plan to study the unfermented pomegranate juice separately, the fermented pomegranate juice from which the seeds were removed prior to fermentation, and also the juice fermented and aged with the seeds inside as was used in this study.

Finally, even though pfj may not in the end be an inhibitor of cyclooxygenase catalyzed prostaglandin formation, it may still have an indirect role to play in inhibition of inflammation, as well as in inhibiting the pathogenesis of more complex disease patterns such as AIDS, carcinogenesis, atherosclerosis and diabetic sequelae through a more general antioxidant effect (Sen and Packer, 1996). A rapidly growing body of work strongly suggests that the overall reduction–oxidation (redox) state in the cytoplasm may itself act profoundly in activating and deactivating certain genes. Specifically, reactive oxygen species (ROS) such as  $H_2O_2$  in high enough concentrations may act as ‘signal transduction messengers’ to promote the activity of at least two factors, nuclear factor NF- $\kappa$ B and activator protein AP-1, whose receptor sites are located on the promotor regions of different genes involved in HIV replication, atherosclerotic mechanisms, carcinogenesis and diabetic changes. In short, suppression of intracellular oxidation significantly reduces the transcription of several key proteins (Barnes and Karin, 1997), including ‘leukocyte-endothelial adhesion molecules’ (Collins et al., 1995), cyclooxygenase (Newton et al., 1997), lipoxygenase and NO synthase. Through this mechanism, as well as via the suppression of lipoxygenase-catalyzed leukotriene formation, pfj and other natural antioxidants may in the end still act as anti-inflammatory agents in addition to their traditional role in preventing the oxidation of lipids.

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