Natural Products Chemistry & Research
ISSN: 2329-6836
Semen et al., Nat Prod Chem Res 2013, 2:1
DOI: 10.4172/2329-6836.1000122
Research Article Open Access

Redox Modulation by Amaranth Oil in Human Lung Fibroblasts

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Abstract

Amaranth oil has several health benefits. It has lipid lowering, anti-diabetic, immune modulatory and cytoprotective properties, activates the function of mitochondria and improves heart rate variability. It has been suggested that the effect of amaranth oil on redox status is involved in this multitude of cellular and clinical influences of the oil.

We examined whether amaranth oil can modify free radical production. EPR experiments with amaranth oil dissolved in DMSO showed scavenging of carbon centered radicals but not of oxygen centered radicals. Moreover, a concentration dependent scavenging effect of amaranth oil on ultrasound-induced radicals was observed. However, in adrenaline autoxidation experiments amaranth oil showed a strong prooxidant action through activation of superoxide anion formation.

This two-sided effect of amaranth oil, i.e. both anti- and pro-oxidant action, was corroborated in human lung fibroblasts that were exposed to amaranth oil. At low concentrations of amaranth oil, fibroblasts were protected against oxidative stress, whereas in incubations with high amaranth oil concentrations more H_2O_2-induced intracellular radical damage was found.

We suggest that mild pro-oxidant activity could be the underlying mechanism in the health beneficial effect of amaranth oil.

Keywords: Amaranth oil; Electron paramagnetic resonance; Adrenalin autooxidation; Antioxidant activity; Redox modulation; Lung fibroblasts

Introduction

Production of Reactive Oxygen Species (ROS) exceeding the capacity of the antioxidant defense with the consequent damage to lipids, proteins and DNA is a well documented cellular event in the development of for example pulmonary, cardiovascular, and neurodegenerative diseases [1]. Over the past several decades, *in vitro* and *in vivo* research provided evidence for a beneficial effect of antioxidants, i.e. compounds able not only to interfere with ROS but also to modify pro-/antioxidant balance, the redox state. However, although effective ROS scavenging activity of certain phytochemicals is well documented in the test tube, protective effects in clinical randomized trials are less clear [2]. Despite these disappointing clinical results, the interest of the researchers in antioxidants sustained. This is primarily due to many observational studies showing good prospective of the dietary antioxidants to provide optimal health [2-5].

ROS as signaling molecules mediating an adaptive response are recognized in health-protective effects of nutritional antioxidants [1]. In fact, mild prooxidant activity can boost the antioxidant defense systems, particularly through Nrf2 signaling [6-10]. In this regard, compounds, which are not only able to scavenge free radicals, but maintain an optimal ROS flow, may upregulate cytoprotective enzymatic antioxidants thus improving health [11,12]. This multi-target paradigm has recently been applied in drug discovery and in dietary supplements studies [3,13].

It has already been affirmed that the oil from *Amaranthus* seeds is a valuable nutraceutical due to its lipid lowering, anti-diabetic, immune modulatory and cytoprotective properties and the ability to activate membrane function and to increase the heart rate variability [14-18]. The diverse health effects of Amaranth Oil (AmO) have been attributed to its specific chemical composition, i.e. a high level of linoleic acid (up to 50%), tocopherols/ tocotrienols and squalene (up to 8%), which take part in redox reactions [19,20]. However, the mechanisms by which AmO protects cells and tissues remain to be established. We hypothesized that AmO salutary effects might be attributed to both antioxidant and a mild prooxidant activity which subsequently affects the intracellular antioxidant defense activity.

The aim of this study was to examine whether AmO can modify free radical production in *in vitro* studies and influence intracellular ROS production. For this study human lung fibroblasts were used because the redox modulation of these cells might be critically important in fibroblast proliferation and function.

Materials and Methods

Materials

Human fetal lung fibroblasts HFL1 (CCL-153) were purchased from American Type Culture Collection (Manassas, VA, USA). The Kaigh's modification of Hams F-12 medium, Fetal Bovine Serum (FBS) was purchased from Gibco (Bleiswijk, the Netherlands). Phosphate Buffered Saline (PBS), sodium pyruvate, Dimethyl Sulfoxide (DMSO), reduced Nicotinamide-Adenine Dinucleotide (NADH), 2,4-dinitrophenylhydrazine (DNPH), trichloroacetic acid (TCA), superoxide dismutase (SOD), hydrogen peroxide, 2′,7′-dichlorofluorescein-diacetate, 5,5-Dimethyl-1-Pyrroline-N-Oxide (DMPO) and triton X-100 were purchased from Sigma (St. Louis,

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Received September 29, 2013; **Accepted** November 28, 2013; **Published** December 05, 2013

Citation: Semen KO, den Hartog GJM, Kaminskyy DV, Sirota TV, Maij NGAA, et al. (2013) Redox Modulation by Amaranth Oil in Human Lung Fibroblasts. Nat Prod Chem Res 2: 122 doi:10.4172/ 2329-6836.1000122

MO, USA). Samples of AmO were provided by the Olivia Company (Kiev, Ukraine).

Plant material and oil composition

The lipid fraction from the seeds of *Amaranthus hypochondriacus L.* was obtained via CO_2 extraction. The fatty acid content of the oil was examined after transesterification under standard alkaline conditions in order to transform the fatty acid residues of triglycerides into their corresponding fatty acid methyl esters (FAME). The analysis was carried out by gas chromatography. The GC equipment (Agilent 6850, Milan) was equipped with a 60m × 0.25mm × 0.25µm (50%-cyanopropyl)-methylpolysiloxane column (DB23, Agilent, USA), and a flame ionization detector with the following oven program: temperature started from 165°C, held for 3 min, followed by an increase of 1°C/min up to 195°C, held for 40 min, followed by a second increase of 10°C/min up to 240°C, and held for 10 min. A constant pressure mode (29 psi) was chosen with helium as carrier gas. Methyl esters were identified by comparison with the retention times of commercially available authentic samples. The fatty acid content is described as relative percentage of each peak area taking into account the total fatty acid peak areas (recognized peaks > 98%).

Electron paramagnetic resonance

Free radical production was monitored using EPR as described by Floyd and Wiseman [21]. Two series of experiments were performed. In the first series production of free radicals was assessed in DMSO dissolved AmO (0.5%, 5.0%) samples under normal and stress conditions. Subsequently, 50 µl of each AmO solution was mixed with 25 µl of 80 mM DMPO and 12.5 µl of 8 mM H_2O_2 and 12.5 µl of 8 mM $FeCl_2$. A DMSO sample (without AmO) served as a control. Additionally stress was applied to the samples by sonication for 10 min, using a Sonorex RK 100 Bandelin GmbH (Berlin, Germany). EPR analysis was performed directly after the sonication at room temperature.

In the second series of EPR experiments radical generation was determined in an adrenalin autooxidation model. To this end 500 µl of 1 M DMPO solution was mixed with 500 µl of AmO solution (0.04 µl per 1 ml 0.2 M bicarbonate buffer, pH 10.5) and this mixture was incubated at 37°C for 1 minute. Subsequently, 15 µl of 0.1% adrenaline hydrochloride was added and mixed. The readings were taken after 45 seconds, 10 min, 15 min, 20 min and 60 min.

EPR spectra were recorded at 9.8 GHz using a Bruker ESR 300 EMX spectrometer (Bruker GmbH, Karlsruhe, Germany) and operating at a center field strength of 3,488 G with 65 G as sweep width, a modulation amplitude of 1 G and microwave power of 10 mW. The time constant and the conversion time were 40.28 and 20.48 ms, respectively, and the results are the average of ten scans.

The scavenging activity (SA) of AmO was defined as $SA=[(h_0-h_x)/h_0]\times100$ (%), where h_0 is the height of the third peak in the EPR spectrum of DMPO radical adducts in the DMSO sample, and hx is the height of the third peak in the EPR spectrum of DMPO radical adducts in the incubations containing AmO.

UV VIS Spectroscopy

The pro-/antioxidant activity of AmO was studied in the adrenalin autooxidation model as described by Misra and Fridovich in modification [22,23]. This method is based on the reaction of the adrenalin autooxidation to adrenochrome in alkaline medium with concomitant production of superoxide anion radical. The pro-/antioxidant activity was assessed by determining changes in the reaction rate. AmO (0.04 µl/ml or 5 µl/ml) was added to 1 ml of 0.2 M carbonate buffer (pH 10.5). Subsequently, 50 µl of 0.1% adrenalin hydrochloride was added and mixed. Finally, 5 µl of 5000 U/ml SOD was added to the sample (final concentration 25 U/ml). The rate of adrenalin autooxidation was determined spectrophotometrically at 347 nm for 7 min. All measurements were performed at 25°C. The adrenalin autooxidation rate in the control vial (without AmO) was assessed under the same conditions. The value of the pro-/antioxidant activity of the sample was monitored by changes of its optical density (absorbance at 347 nm) during 7 minutes and was calculated by $A=[(E_t-E_1)study/(E_t-E_1)_{control}]\times100\%$, where E_1 – initial absorbance after introduction of adrenalin; E_t – absorbance at the time of reaction termination. Inhibition of the reaction is interpreted as antioxidant effect whereas stimulation is a reflection of the prooxidant effect.

Cell culture

Human fetal lung fibroblasts HFL1 (CCL-153) were cultured in Kaigh's modification of Hams F-12 medium supplemented with 10% FBS and 1% Penstrep and were maintained at 37°C in a humidified incubator (5% CO_2). All experiments were done on CCL-153 passages four and five.

Measurement of intracellular ROS using 2',7'-dichlorofluorescein (DCFH) assay

The fluorescent probe 2',7'-dichlorofluorescein-diacetate was used to quantify intracellular oxidative stress in CCL-153 cells. This nonpolar probe crosses cell membranes and is enzymatically hydrolyzed by esterases to DCFH which is trapped in the cell. In the presence of ROS, the non-fluorescent DCFH is oxidized to the highly fluorescent form DCF. The intensity of fluorescence corresponds to the level of intracellular ROS [24-26].

First, lung fibroblasts were exposed to several concentrations AmO dissolved in DMSO, i.e. 0%, 2.5×10^{-8}%, 2.5×10^{-6}%, 2.5×10^{-4}% or 2.5×10^{-3}% for 40 minutes (acute exposure), 24 hours or 72 hours without refreshing the medium. The concentration DMSO in the medium was held constant at 0.05%. The maximal concentrations of AmO was determined by it solubility in DMSO. After this pretreatment period, fibroblasts were seeded in 96-well plates at a concentration of 10^4 cells/well and grown for 24 hours. On the next day 50 µl 60 µM 2′,7′-dichlorofluorescin-diacetate was added to the cells and incubated for 45 min at 37°C, 5% CO2. Medium was removed and cells were washed with HBSS to remove extracellular 2′,7′-dichlorofluorescin-diacetate. Later CCL-153 were treated with HBSS or with 100 µM H_2O_2. Fluorescence was measured in each well every 2 min with excitation and emission wavelengths of 485 and 538 nm, respectively, using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 37°C over a period of 46 min. Experiments were repeated in seven wells.

Lactate dehydrogenase (LDH) assay

Cell death was determined with the LDH assay using pyruvate as substrate [27] After pretreatment with AmO, CCL-153 cells were incubated with either HBSS (blank) or 100 µM H_2O_2. Subsequently incubation media were collected 200 µl of a reaction mixture consisting of 1.125 mg/ml sodium pyruvate and 0.9 mg/ml NADH was added. LDH activity was measured spectrophotometrically at 340 nm for 1 hour. LDH leakage was calculated by the formula: LDH leakage=(LDH activity in medium/total LDH activity)×100%. Lysed cells were used as a reference for 100% LDH leakage.

Statistical analysis

Data are shown as means ± S.E.M. Differences between means were

tested for significance using unpaired Student's t-test. Differences were considered statistically significant at p-values <0.05.

Results

Composition of AmO

The main ingredients of AmO include linoleic acid ($C_{18:2}$) 56.97%, oleic acid ($C_{18:1}$) 22.13%; palmitic acid ($C_{16:0}$) 10.20%, and also squalene 3.7%. Various other fatty acids were found including small amounts of the ω-3 ($C_{18:3}$) - α-linoleic acid (Table 1).

Influence of AmO on free radical formation

EPR experiments with DMPO as spin trap did not demonstrate any ROS production in samples with 0.5% and 5% concentrations of AmO dissolved in DMSO. Addition of H_2O_2 and Fe^{2+} to the mixtures induced the Fenton reaction and triggered formation of both carbon centered radicals (a_H: 14.0 G & a_N: 14.5 G) and oxygen centered radicals (a_H: 22.0 G & a_N: 16.3 G). These radicals were most prominent in the control DMSO containing sample (Figure 1a). AmO caused a concentration dependent decrease in carbon centered radicals (0.5% AmO, SA=70%; 5% AmO, SA=86%) but did not influence the amount of oxygen centered radicals (Figure 1b and 1c).

Sonication of DMSO containing samples triggered a specific DMPO EPR signal which was reduced by administration of 0.5% AmO and 5% AmO with a SA of 62% and 50%, respectively at the initial phase (Figure 2a-2c).The same radical pattern was observed initially, 5 and 10 min after stress for each sample series (only direct measurements are shown).

In the EPR experiments using the adrenaline auto oxidation model addition of adrenaline to DMPO resulted in a time-dependent but transient increased DMPO (Figure 2). In the presence of 0.004% AmO, the baseline DMPO OH signal was lower compared to the control and no increase was observed. It remained at basal level over time. This is indicative for a radical scavenging activity of AmO and possibly maintenance of some DMPO OH signal level during the time of experiment.

To the contrast the UV-VIS spectroscopy study showed that after addition of AmO the rate of adrenaline autooxidation more than doubled (Figure 3a). Addition of SOD1 (10 U.ml-1) completely prevented this increase and returned the rate of adrenochrome formation to below baseline level. Similar results were obtained with higher (0.5%) concentrations of AmO (Figure 3b). Even more, the addition of SOD to the control samples led to a decrease of the autoxidation rate. It is a clear indication for superoxide anion formation under AmO action. In agreement with the EPR experiments more pronounced effect were observed at lower AmO concentrations.

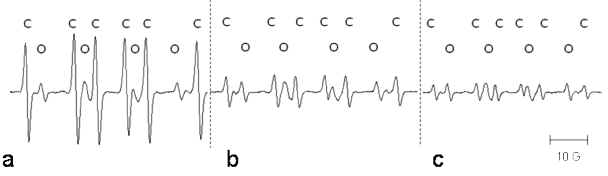

Figure 1: Radical scavenging by amaranth oil.Radicals were induced with H_2O_2 and $FeCl_2$. a=DMSO (control). b=0.5% AmO c=5% AmO. Two types of radicals can be distinguished: C - carbon centered radicals (with splitting factors: A_H: 22.000 & A_N: 16.250) and O - oxygen centered radicals (splitting factors: A_H¬: 14.0 G & A_N: 14.5 G).

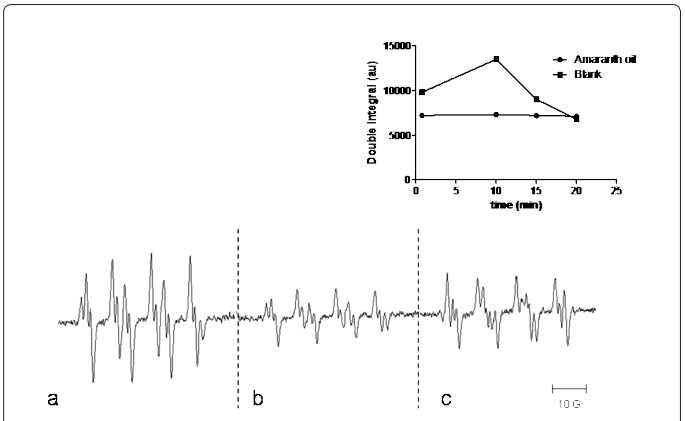

Figure 2: Scavenging of ultrasound-induced radicals by amaranth oil in a DMPO (320 mM) solution. The DMSO containing sample after sonication demonstrated a specific DMPO signal (a), which was reduced with administration of 0.5% AmO (b) and 5% AmO (c). Right upper corner: changes of the DMPO radical adduct formation in an adrenalin autooxidation system by (4×10^{-3}%) AmO. The total radical production was quantified by computing the double integral of the EPR absorption spectrum.

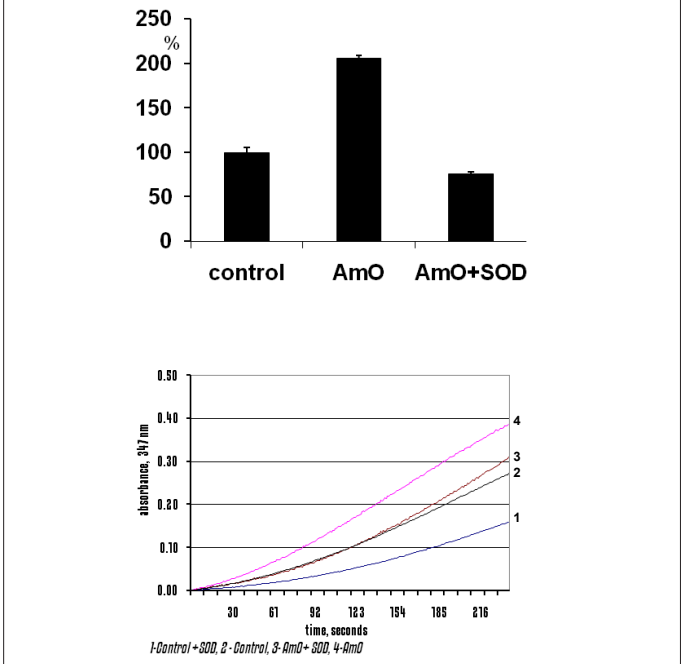

Figure 3: (a) Effect of amaranth oil (0.004%) alone or in combination with SOD1 (10 U/ml) on adrenaline autooxidation.
(b) An example of amaranth oil (0.5%) induced increase in absorbance during adrenaline autooxidation and inhibition by SOD1.

Intracellular ROS production

Preincubation of the fibroblasts with different concentrations of AmO changed the intracellular ROS production under stress conditions (H_2O_2) (Figure 4a and 4b). Although there were no significant changes after the acute (40 min) exposure to AmO, low AmO concentrations (2.5×10^{-8}%) tended to decrease the rate of H_2O_2-induced intracellular ROS production, while higher AmO concentrations increased intracellular ROS formation. After 24 hours of AmO preincubation a slight decrease in the rate of H_2O_2-induced ROS production was observed but only in the AmO 2.5×10^{-4}% exposure group a significant

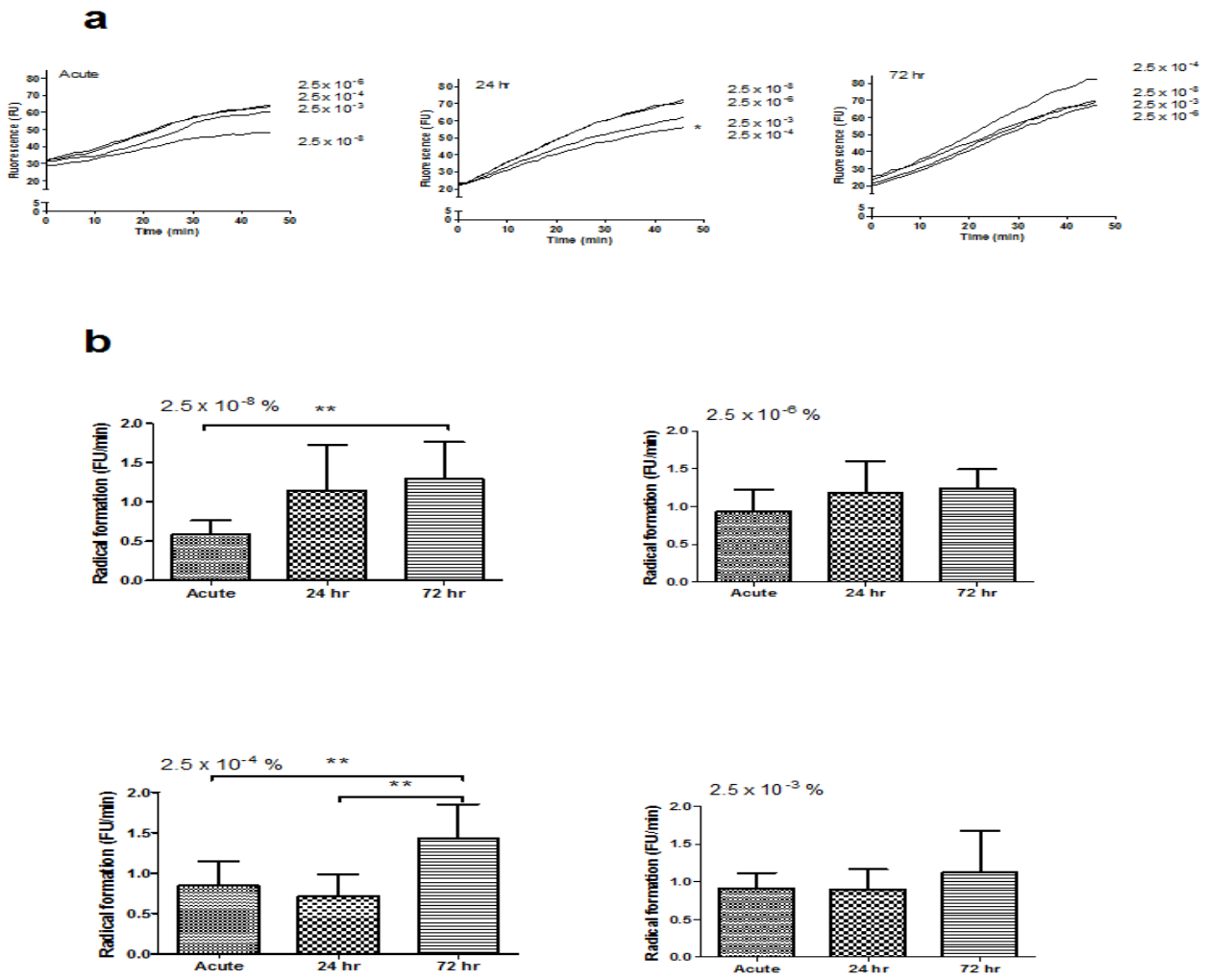

Figure 4: Intracellular ROS production after incubation of fibroblasts with amaranth oil and subsequent exposure to 100 μM H_2O_2. (a) DCF fluorescence traces directly after addition of AmO or after 24 hr and 72 hr incubation with AmO. (b) Effects of different concentrations of AmO on DCF formation.

decrease (p=0.013) was noted. Finally, incubation of the fibroblasts with the AmO for 72 hours did not show any significant differences compared to DMSO and control incubation (Figure 4a).

Exposure to different concentrations of AmO caused a time-dependent increase in H_2O_2-induced ROS production in all groups (Figure 4b) Longer AmO preincubation periods were associated with increased H_2O_2-induced intracellular radical production. Especially when the 72 hours group is compared to the acute exposure a significant increase in the 2.5×10^{-8}% (Figure 4b, p=0.0016,) and 2.5×10^{-4}% (Figure 4, p=0.0075) concentration groups was seen. Furthermore, when comparing 24 to 72 hours AmO pre-exposures a significant increase (p=0.0043) in the AmO 2.5×10^{-4}% group was noted. Interestingly, no significant changes in H_2O_2-induced free radical production after the pretreatment with the highest concentration of AmO were observed.

Cell viability

The direct cytotoxic effect of various incubation conditions was tested by a cell viability study (Figure 5). Pretreatment with various concentrations of AmO prevented H_2O_2 induced increase in cell damage. The low (2.5×10^{-6}%) concentration of AmO slightly increased LDH leakage compared to the control, but in combination with H_2O_2 markedly improved cell survival was observed.

Discussion

Plant oils have already received much attention as source of antioxidants but the unique composition of AmO makes it an outstanding natural product for the prevention and treatment of Reactive Oxygen Species (ROS)-related pathologies [28]. It has been shown that amaranth grains have a moderate, protective effect against the changes promoted by fructose-induced oxidative stress in rats through reduction of lipid peroxidation and increasing the antioxidant capacity of tissues [29]. In patients with coronary vascular disease, use of AmO was associated with lowering of the products of lipid peroxidation with concomitant increase in glutathione reductase, glutathione peroxidase, superoxide dismutase, and catalase activities [30]. Moreover, the consumption of AmO was shown to decrease signs of oxidative stress and ameliorate heart rate variability in diabetic patients and to improve the adaptive potential in athletes [19].

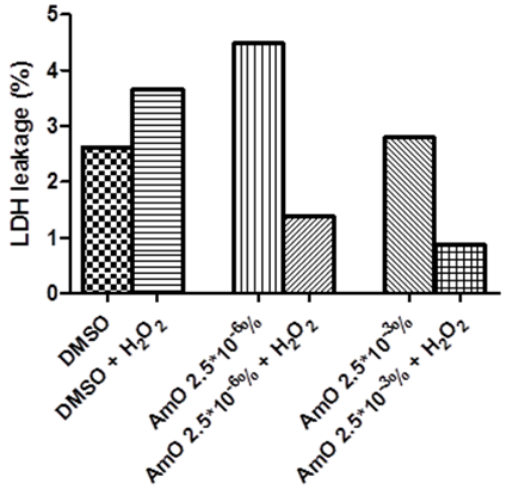

Figure 5: Amaranth oil associated changes in LDH leakage. LDH leakage in control was 3.8%; in control under stress conditions - 7.3%.

Compound			%
Fatty acids			
	linoleic	18:2 (9c,12c)	57.0
	Oleic	18:1 (9c)	22.1
	palmitic	16:0	10.2
	stearic	18:0	3.9
	vaccenic	18:1 (11c)	0.76
	behenic	22:0	0.74
	α-linoleic	18:3	0.50
	arachidic	20:0	0.49
	lignoceric	24:0	0.25
	eicosenoic	20:1	0.20
	myristic	14:0	0.12
	palmitoleic	16:1	0.10
	heptadecanoic	17:0	0.06
	Total fatty acids		96.3
Squalene			3.7

Table 1: Composition of amaranth oil.

In an attempt to shed more light on the mechanism by which AmO exerts its biological activity first its composition should be discussed. Interestingly, the main fatty acids (palmitic, linoleic and oleic acid), which were found in AmO (Table 1) possess a rather prooxidant behavior [31,32]. The high level of the mono-unsaturated oleic acid is interesting because of its beneficial effect on the cell membrane by increasing fluidity and decreasing peroxidation index [33]. Their effect can be counter balanced by other AmO constituents, particularly, vaccenic acid, α-linoleic acid, squalene and minor components (tocopherols, tocotrienols). Indeed, although only present in a small fraction (0.8%), vaccenic acid can be of significance because after biotransformation into conjugated linoleic acid (9-*cis*,11-*trans* $C_{18:2}$), it can serve as an efficient ligand for the peroxisome proliferator-activated receptors [34] and thus mediate the ω-3 mimetic activity of AmO. Together with stimulation of the peroxisomal oxidation AmO was demonstrated to activate the energy function of liver mitochondria in the rat and to prevent their hyperactivation under adrenaline stress, thus, proving AmO induced stress protection [16] Squalene quenches singlet oxygen and prevents oxidative DNA damage in human mammary epithelial [35]. Interestingly, squalene has very low antioxidant activity compared to the lipophilic extract of amaranth seeds [12]. Thus, it can be hypothesized that the composition of pro- and antioxidant compounds found in AmO can induce the adaptive stress response also known as a hormetic reaction, which could be responsible for its beneficial biological effects.

In this study we investigated the possible combined radical scavenging and generating activity of AmO. To this end AmO was dissolved in DMSO and exposed to hydroxyl radicals, which were generated by Fe^{2+} and H_2O_2, the so-called Fenton reaction. Using EPR we demonstrated that DMSO in the Fenton mixture gave rise to oxygen and carbon centered radicals (Figure 1a). AmO faded the carbon-centered radicals in a concentration-dependent manner but not the oxygen-centered radicals (Figure 1b and 1c). This clearly points to the scavenging action of AmO.

When radicals were generated by ultrasound in the presence of the spin-trap molecule DMPO, the scavenging action of AmO is straightforward (Figure 2). The antioxidant action of AmO could also be demonstrated in the adrenaline autoxidation reaction (Figure 2). It should be noted that in the EPR-ultrasound experiments, the low concentration of AmO (Figure 2b) is more effective than the high concentration (Figure 2c). This concentration dependency of AmO effects made us decide to study the effect of AmO also in low concentrations in the adrenaline autoxidation assay. Remarkably, also in this assay, a low concentration of AmO (0.004%) accelerated the autoxidation of adrenaline (Figure 3). Superoxide anion radicals are known to be involved in this stimulation [23,36] and also in our experiments the AmO mediated acceleration could be prevented by SOD (Figure 3). Similar but less prominent effects were found in the experiments using higher AmO concentrations. Thus, AmO shows both anti- and pro-oxidant behavior.

The above experiments were all performed under oxidative stress conditions, viz. H_2O_2/Fe^{2+}, ultrasound and adrenaline autoxidation system. Therefore it was decided to study the effects of AmO pretreatment in lung fibroblasts before oxidative stress is applied. Production of TGFb and collagen by lung fibroblasts is initiated by oxygen radicals [37,38]. Consequently, the antioxidant action of AmO might prevent the fibrotic response of lung fibroblast after an oxidative insult. Moreover, the antioxidant action of AmO might be direct or indirect via time dependent activation of an adaptive antioxidant response. The latter might be the result of a mild prooxidant action of AmO.

In an attempt to get hold on these apparent counteracting processes, fibroblasts were incubated with various concentrations of AmO and for different incubation periods. The fibroblast viability measurements clearly demonstrated a stress protective action of AmO (Figure 5). These findings were accompanied by mild prooxidant action in all tested conditions as it was shown by EPR experiments (Figure 4) which is suggestive for a hormetic/parahormetic mechanism involved in the AmO effects [10,11].

Thus, AmO was demonstrated both to activate and to inhibit free radical flow in *in vitro* studies. Scavenging activity of AmO was noted more prominently towards to C centered radicals, at the same time prooxidant effects of AmO were possibly related to O centered radicals. Such modulatory effects on free radical metabolism were more significant for low concentrations of AmO and were could account for the cytoprotective and stress protective action of AmO in fibroblasts. Mild prooxidant activity could be the underlying mechanism involved in the health beneficial effects of AmO.

Acknowledgement

The authors acknowledge Olivia Company (Kharkiv, Ukraine) for kindly providing concentrated Amaranth oil for investigation. Also we would like to express our gratitude to Dr. Carla Ferreri and Dr. Chryssostomos Chatgilialoglu (ISOF-Consiglio Nazionale delle Ricerche, Bologna, Italy) for their contribution to this study, especially, for the determination of Amaranth oil composition.

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