



Effects of the Dietary Supplement MAK4 on Oxidative Stress Parameters: A “Three-Cases” Report

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Abstract

Background: Maharishi Amrit Kalash-4 (MAK-4) is an herbal formulation comprised of a several herbs. This preparation is part of a natural health care system known as Maharishi Ayur-Veda and belongs to a series of herbal mixtures collectively known as Rasayanas, which are recognized for their beneficial effect on human health. This investigation evaluated in blood samples of 3 human volunteers the effects of MAK-4 on parameters of oxidative stress and antioxidant defenses. **Methods:** The day of the experiment: 3 subjects were put on the following diets: Diet 1: 300 gr boiled vegetables (zucchini and potatoes) and 100 gr of wheat bread (control); Diet 2: 300 gr raw vegetables (lattice, carrots, cavolflovers) and 100 gr of wheat bread; Diet 3: diet 1 plus 500 gr of oranges; Diet 4: diet 1 plus MAK-4 (30 gr/day). **Short term treatments:** The same subjects have been on the four different diets on alternative days. Meals were at 12 am and 6:30 pm. MAK-4 (10 gr each intake) was assumed three times at 9 am, 12 am and 6:30 pm (total 30 gr). Blood was withdrawn three times, at 9 am (T = 0), just before the first intake of MAK-4 (first), at 7 pm (second) and at 9 am, the following day (third). **Long term treatments:** subjects were on a free personal diet for 60 days (control). For the following 60 days the subjects were on the same diet plus 20 gr of MAK-4/day. MAK-4 (10 gr) was assumed twice a day, at 9 am and at 6 pm for 60 days. Blood was withdrawn at the beginning of the study (T = 0) and after 60 days, at the same day time (9 am). **Results:** In the short term study MAK-4 induced two endogenous parameters of antioxidant defenses: Oxygen Radical Absorbance Capacity (ORAC) and total thiols. Peak induction was observed at 9 hours after the assumption of the first intake. Up-regulation of ORAC was similar to that obtained with 500 grs of oranges although with MAK-4 it remained sensibly higher than the control at 24 hours. Thiols maximally increased with MAK-4 at 9 hours, but returned to basal levels at 24 hours. Exogenous intake of ascorbic acid contained in MAK-4 showed a maximal level at 9 hours, although generally lower than that assumed with 500 gr of oranges. At 24 the ascorbic acid from MAK-4 was at the same level of that assumed with oranges. The same parameters measured in

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blood of the subject on Diet 2 showed a slight increase at 9 hours, although it was significant only for ascorbic acid. In the long term study the MAK-4-supplemented diet caused a decrease in a parameter of oxidative load (hydroperoxides) and an upregulation of ORAC, thiols and ascorbic acid. The same subjects did not show the same variations when they were on the basal diet alone. **Conclusions:** Our results show that a MAK-supplemented diet decreases oxidative stress parameters and increase antioxidant defenses in both short and long term treatments.

Keywords

Food Supplements, Herbs, Oxidative Stress, Antioxidants

Subject Areas: Biochemistry, Food Science & Technology, Nutrition

1. Background

The herbal food supplement Maharishi Amrit Kalash-4 (MAK-4) is a formulation of herbal mixtures. This preparation is part of a natural health care system from India, known as Maharishi Ayur-Veda [1] [2]. A large body of research has been published on this herbal mixture, showing its beneficial properties for health [3]-[8]. MAK-4 was shown to prevent 7,12-dimethylbenz(a)anthracene-induced mammary carcinoma [8] [9] and aggressive lung cancer in rats and to increase levels of mRNA transcripts of two genes that code for hepatic glycosyltransferases in the rat hepatic nodules from which cancer arises [10]. MAK-4 is often used in combination with another herbal formulation called MAK-5 that has also anticancer properties. *In vitro* experiments showed that MAK-5 produced morphological and biochemical differentiation of 75% of mouse neuroblastoma cells in culture [11], potentiate NGF inducing neuronal differentiation in PC12 cells [12] and inhibited processes of neoplastic transformation in rat tracheal epithelial cell lines and human lung tumor cell lines [13].

Recently we evaluated the cancer inhibiting effects of both MAK-4 and MAK-5 *in vitro* and *in vivo* [3]. These mixtures were found to inhibit cell transformation induced by the *ras* oncogene *in vitro* and inhibit liver carcinogenesis in mice. The effect of MAK-4 and MAK-5 on carcinogenesis may be due to several different mechanisms such as the ability to scavenge free radicals and reactive oxygen species (ROS) and regulate connexins levels which are markers of tissue integrity and tumor formation.

The antioxidant properties of MAK-4 and MAK-5 were confirmed in mice in which the oxygen radical absorbance capacity (ORAC) was significantly higher following a diet supplemented with MAK. Endogenous antioxidants defence such as the liver enzymes GPX, GST and QR and connexins expression were also strongly stimulated in the MAK-fed mice. The loss or the alteration of gap junction intercellular communication (GJIC) has long been proposed to play an important role in the process of carcinogenesis [14]. Penza *et al.* [3] postulated that the increase antioxidant defenses and liver-specific connexin expression, may be involved in the mechanism of action of the anticancer potential of these natural formulations.

Free radicals have been implicated in carcinogenesis [15]-[19]. Antioxidants, which provide protection against free radical damage, are present in MAK 4 [20]. The chemical composition of these herbal mixtures includes alpha tocopherol, beta-carotene, ascorbate, bioflavonoid, catechin, polyphenols, *trans* resveratrol, riboflavin, tannic acid, and other low molecular weight substances [21].

In this work we tested the antioxidant properties of a standardized preparation of MAK-4 in the serum of subjects on a MAK-4 supplemented diet and compared its effect to that observed in subjects on 500 grams of oranges.

2. Materials

2.1. Chemicals

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and 1-chloro-2,4-dinitrobenzene were obtained from Aldrich Chemical Co. (Milan, Italy). The Chelex 100 resin (200 - 400 mesh, Na form) was purchased from Mallinckrodt Inc. (St. Louis, Mo, USA). The Ransel kit was obtained from Randox (Crumlin, UK). All the other reagents were obtained from Sigma Chemical Co. (Milan, Italy) unless otherwise indicated.

2.2. Herbal Mixture

Maharishi Amrit Kalash-4 (MAK-4) and -5 (MAK-5) were gifts from the Global Trading Group (Verona, Italy). MAK-4 is in paste form and contains the following ingredients: Raw sugar, Ghee, *Terminalia chebula*, *Embllica officinalis*, *Hooney*, *Desmodium gangeticum*, *Tribulus terrestris*, *Centella asiatica Urban*, *Cinnamon*, *Cyperus rotundus*, *Curcuma longa*, *Piper longum*, *Santalum album*, *Bacopa monnieri*, *Cyperus scariosus*, *Cyperus rotundus*, *Elettaria cardamomum Maton*, *Butea monosperma*, *Glycyrrhiza glabra*, *Mesua ferrea*, *Convolvulus pluricaulis*, *Embelia ribes*, *Aegle marmelos*, *Correa*, *Asparagus racemoso*, *Clerodendron phlomidis*, *Desmostachya bipinnata*, *Gmellina arborea*, *Ipomea digitata*, *Leptadenia reticulata*, *Oroxylum indicum*, *Pedaliium murex*, *Phaseolus trilobus*, *Pueraria tuberosa*, *Saccharum officinarum*, *Saccharum spontaneum*, *Solanum indicum*, *Solanum xanthocarpum*, *Stereospermum maveolens*, *Teramnus labialis*.

Qualitative chemical analysis has revealed that MAK-4 contain multiple antioxidants, including alpha-tocopherol, beta-carotene, ascorbate, bioflavonoid, catechin, polyphenols, riboflavin, tannic acid, and other low molecular weight substances [19]. The quantitative analysis of these constituents has not been done. The total polyphenols were assessed by the reduction of phosphotungstic phosphomolybdic acids (Folin Ciocalteu's reagent) by phenols in alkaline solution and sodium carbonate method. Their concentration in crude dried material is 21.76 mg per gram. MAK-4 contains 7 µg per dried gram of trans-resveratrol determined by high performance liquid chromatography. The total antioxidant activity of MAK-4, measured by a modification of the Ferric Reducing Ability of Plasma (F.R.A.P) assay is 144 µmol per gram of dried crude material. The F.R.A.P assay measures the percentage of FeIII (FeCl₃.6H₂O) reduced to FeII by water extractable antioxidants. The production of MAK-4 is subjected to stringent quality control measures to assure uniform composition and quality from batch to batch.

2.3. Subjects

Three adults were volunteers for the study. One woman 45 years old, fertile age, non smoker (subject 1), one woman 32 years old, smoker on birth control therapy (subject 2), one man 35 years old (smoker) (subject 3).

2.4. Determination of Oxidative Stress Parameters

Sera from subjects were analyzed for the levels of the following parameters:

Reactive Oxygen Species: Were calculated by the dROMs kit (Diacron, Grosseto) according to the manufacture indications.

Thiols were assayed by the use of the Ellman's method. This method quantify the thiols with 5,5'-dithio-bis(2-nitrobenzoic acid; DTNB), Ellman's reagent.

Ascorbic acid. The quantitative determination of ascorbic acid in sample was done by the use of ascorbate oxidase which catalyzes the reaction of reduced ascorbic acid to oxidized ascorbic acid and hydrogen peroxide. Ascorbic acid in the sample is reacted with oxygen in the presence of the ascorbate oxidase, chromogen and peroxidase. Absorbance is determined and compared with a known calibration curve.

Oxygen radical absorbance capacity (ORAC). The sera were analyzed for oxygen radical absorbance capacity (ORAC). The reaction mixture for the ORAC assay contained 1.67×10^{-8} M beta-Phycoerythrin (b-PE), 0.3% H₂O₂, and 9×10^{-6} M CuSO₄ in 7.5×10^{-2} M phosphate buffer, pH 7.0 (200 ml). The phosphate buffer was passed through Chelex 100 resin before it was used to prepare the solutions, and was used as a blank in the assay. The reaction mixture and sera were pipetted into fluorimetry 96-well microplates. Trolox, a water-soluble vitamin E analogue, was used as a control standard. Samples containing b-PE alone (1.67×10^{-8} M in phosphate buffer) were also prepared to monitor the spontaneous decay of fluorescence of this indicator protein under the experimental conditions. H₂O₂ and CuSO₄ were added to each well to generate hydroxyl radicals (OH[•]), then the microplate was put into the pre-warmed plate (37°C) of a Titertek Fluoroscan II (Flow Laboratories, Milan, Italy) and the fluorescence of b-PE was measured every five minutes until zero fluorescence occurred, using the excitation and emission wavelengths of 540 nm and 565 nm, respectively. All fluorescence readings were automatically corrected for the spontaneous decay of b-PE. The ORAC values of the sera were calculated by measuring the net protection area (S) under the quenching curve of b-PE in the presence of the sera. One ORAC unit was designated as the net protection provided by 1 mM (final concentration) Trolox. The ORAC units of a sample were calculated as follows: ORAC units = (Ssample - Sblank)/(S1mM Trolox - Sblank).

3. Results and Discussion

Effect of MAK-4 on parameters of oxidative stress and antioxidant defenses.

In the short term study (Figure 1) MAK-4 induced two endogenous parameters of antioxidant defenses: Oxygen

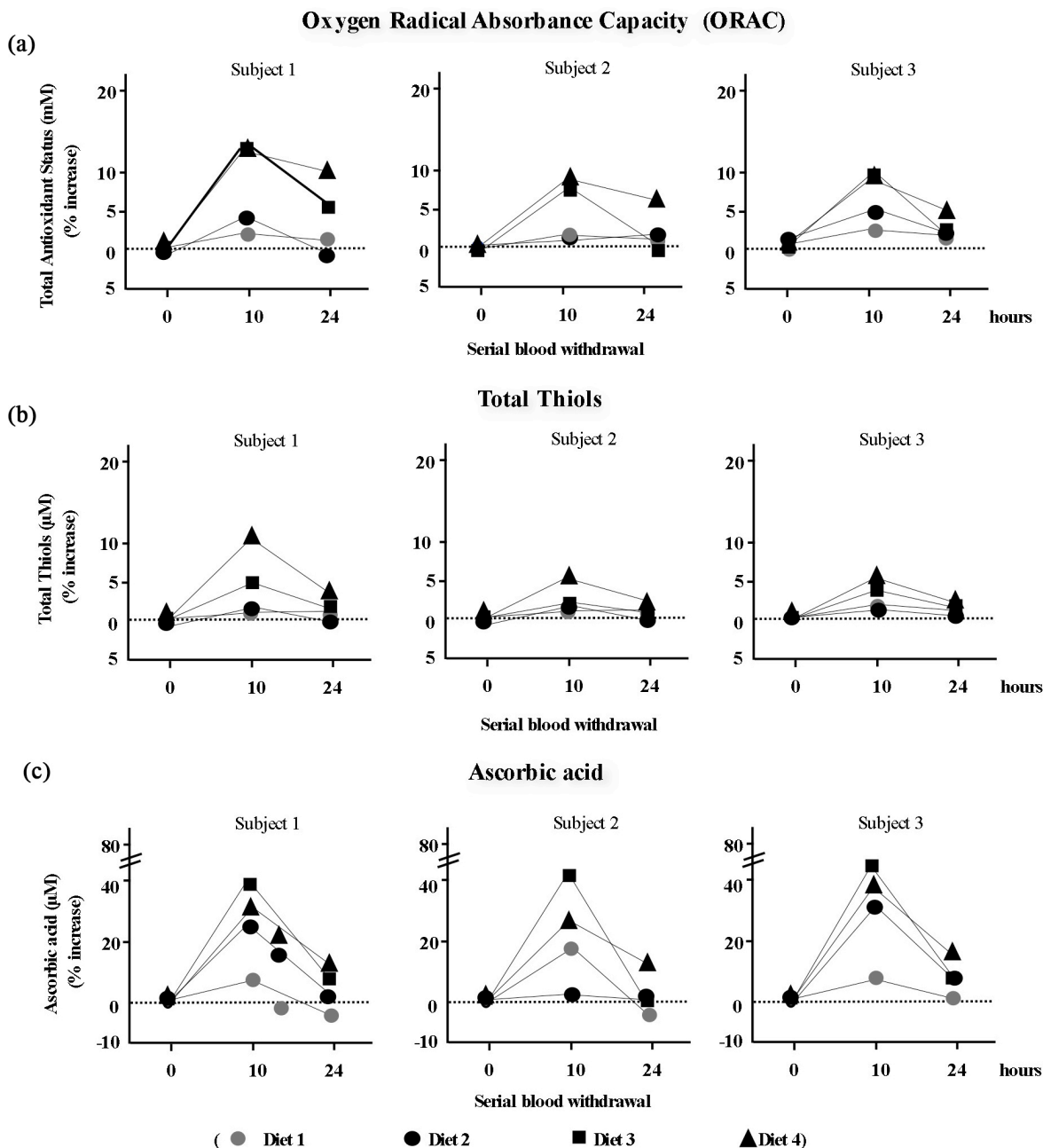


Figure 1. Effects of MA4 assumption on biochemical parameters of oxidative stress. Short term analysis. The biochemical parameters indicated in the figure (Oxygen Radical Absorbance Capacity (ORAC), Total thiols (TT) and Ascorbic acid (AA), have been measured in 3 adult subjects before and after the assumption of MA4 at the following times: 10 gr at 9.00 am, 10 gr. at 12:30 am, and 10 gr. at 5:30 pm. The day of the experiment the 3 subjects were on the following diets: Diet 1: 300 gr boiled vegetables (zucchini and potatoes) and 100 gr of wheat bread (control). Diet 2: 300 gr raw vegetables (lettuce, carrots, cavolfowers) and 100 bread. Diet 3: diet 1 plus 500 gr of oranges. Diet 4: diet 1 plus MAK-4. Meals were at 12 am and 6:30 pm. MAK-4 (10 gr each intake) was assumed three times (total 30 gr), at 9 am, 12 am and 6:30 pm. Blood was withdrawn three times, at 9 am (T = 0), just before the first intake of MAK-4 (first), at 19.00 (second) and at 9 am the following day (third) (24 hours).

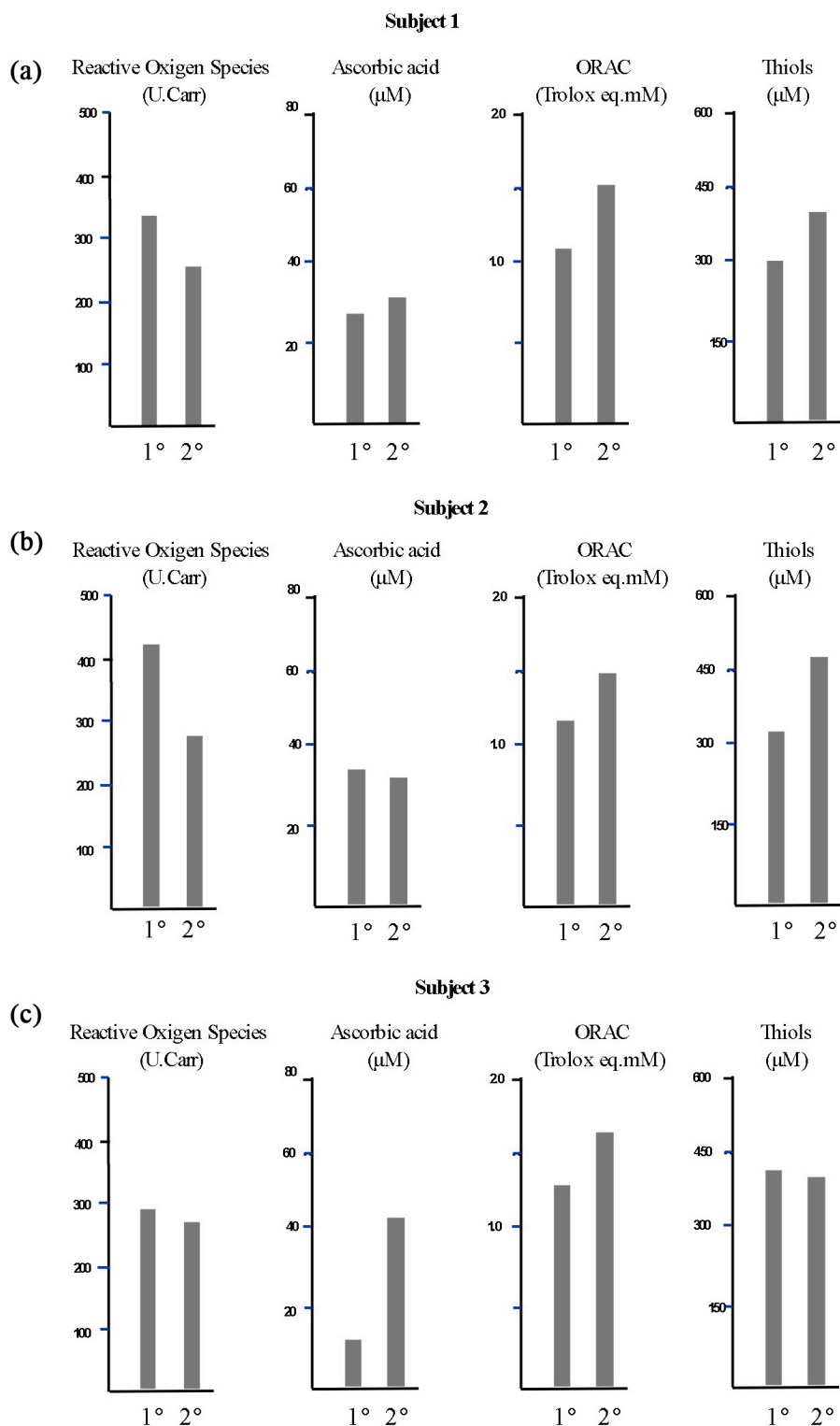


Figure 2. Effects of MA4 assumption on biochemical parameters of oxidative stress. Two months analysis. The biochemical parameters indicated in the figure: Reactive Oxygen Specific (dROMs); Oxygen Radical Absorbance Capacity (ORAC); Total thiols (TT) and Ascorbic acid (AA), have been measured in 3 adult subjects before and after the daily assumption of MA4: 10 grs at 9 am and 10 grs at 6 pm for two months. The subjects were on a free personal diet. MAK-4 (10 gr) was assumed twice a day, at 9 am and at 6 pm for 60 days. Blood was withdrawn at the beginning of the study (T = 0) and after 60 days, at the same day time (9 am).

Radical Absorbance Capacity (ORAC) and total thiols. Peak induction was registered 9 hours after the assumption of the first intake. Up-regulation of ORAC was similar to that obtained with 500 grs of oranges at 9 hours, but remained sensibly higher at 24 hours. MAK-4 maximally increased thiols, but returned to basal levels at 24 hours. The intake of ascorbic acid contained in MAK-4 was also measured and showed a maximal level at 9 hours. It was generally lower than that assumed with 500 gr of oranges, although at 24 it was at a higher level. The same parameters measured in blood of the subject on Diet 2 (raw vegetables) showed a slight increase at 9 hours only for the ascorbic acid.

In the long term study the MAK-4-supplemented diet caused a decrease in a parameter of oxidative load (dROMs) and an upregulation of ORAC, thiols and ascorbic acid (**Figure 2**). The same subjects did not show the same variations when they were on unsupplemented diets for two months before the beginning of the study.

These results show an average increase of the antioxidant defences measured as ORAC and total thiols in the three examined subjects both after assumption of MAK-4 and 500 gr of oranges. It is relevant to observe the higher persistency of the total antioxidant activity in the subjects on MA4 respect to those on 500 gr of oranges.

The results indicate an average increase in the level of ascorbic in the three subjects examined both after assumption of MA4 as well as 500 gr of oranges.

The present study reveals the ability of MAK-4 to scavenge free radicals and reactive oxygen species and to increase antioxidant defenses in human subjects.

Antioxidants which act to control the oxidative state may represent a major line of defense against several diseases. Multiple antioxidants are present in MAK-4 (*i.e.* alpha-tocopherol, beta-carotene, ascorbate, bioflavonoid, catechin, polyphenols, riboflavin, and tannic acid) [21] and previous research has shown that this herbal preparation scavenges free radicals and ROS in a dose-dependent manner [3]. The free radicals and ROS scavenged by MAK-4 include superoxide, hydroxyl, and peroxy radicals, and hydrogen peroxide generated in both cellular (neutrophil) and noncellular (xanthine-xanthine oxidase) systems [22], thus reducing lipid peroxide levels and inhibiting oxidation of low-density lipoprotein (LDL) [23]-[25].

In a recent work, the antioxidant properties of MAK-4 were confirmed *in vivo* in mice [3]. The oxygen radical absorbance capacity (ORAC) assay measured a significantly higher value in mice fed a MAK supplemented diet which increased progressively from day 7 and reached a maximum level at 1 month remaining higher throughout the course of the experiment. Endogenous antioxidant defenses such as the liver enzymes GPX, GST and QR were also stimulated by MAK-4.

Substantial evidence has accumulated to suggest that the induction of phase II enzymes such as GST and QR is a causal mechanism for cancer protection since these enzymes divert ultimate carcinogens from reacting with critical cellular macromolecules. Epidemiological evidence links a diet rich in antioxidants from fruits and vegetables with a low risk of cancer [26], thus natural compounds and natural food supplements may be of help to reach an effective antioxidant protection [27]. The same study also hypothesized that the observed up-regulation of connexins expression by MAK might be considered as a possible mechanism for the cancer-preventive properties of this herbal mixture.

Literature data also indicate other possible mechanisms for the anticarcinogenic properties of MAK-4. Animals treated with MAK-5 have shown a 32% - 88% increase in mitogen-induced lymphocyte proliferation [28], MAK-5 induces *in vivo* priming of both T cells and macrophages, resulting in enhanced functioning of the immune system [5]. In another report the authors showed that MAK-4 has similar immune-enhancing properties as MAK-5 [6]. In addition, MAK-4 significantly increased stimulated splenic production of interleukin-2, which is involved in regulation of the immune system. Altogether these studies indicate that beyond acting as a potent antioxidant, the anticarcinogenic activities of MAK may be also a result of enhanced functioning of the immune system.

4. Conclusion

The present study demonstrates that MAK-4 increases different lines of antioxidant defense in people, shortly after single administrations (30 gr/day) and that the effect was maintained when the daily diet was supplemented with 20 gr/day, for 60 days.

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Competing Interests

The author(s) declare that they have no competing interests.

Authors' Contributions

RDL: subjects recruitment and data evaluation.

DDL: data evaluation.

IZ: oxygen radical adsorbent capacity assay, ascorbic acid, thiols and reactive oxygen species.

All authors read and approved the final manuscript.

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