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BIOLOGICAL EFFECTS OF BIOACTIVE COMPONENTS AND EXTRACTS DERIVED FROM EDIBLE PLANTS COMMONLY USED IN HUMAN NUTRITION

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Biological effects of bioactive components and extracts derived from edible plants commonly used in human nutrition

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ABSTRACT

The main aim of this PhD research project was the evaluation of the biological effects of bioactive compounds derived from edible plants, with particular attention on their possibility to counteract oxidative damage and inflammation.

After a preliminary study of *in vitro* antioxidant activity, regarding the modification eventually occurring after home freezing and cooking of edible vegetables, cultured mammalian cells were used as experimental model systems.

Soluble extract and essential oils derived from different cultivars of Brassicaceae and Lamiaceae were tested as possible tools for the counteraction of the oxidative damage due to reactive oxygen species (ROS), underlining differences related to cultivar and agronomic techniques.

Since accumulating evidence indicates that phytochemicals exhibit several additional properties in complex biological systems, a nutrigenomic approach was used to further explain the biological activity of a green tea extract, and to evidence the anti-inflammatory role of bioactive compounds derived from different foods.

Overall, results obtained could contribute to a better understanding of the potential health benefit of plant foods.

Keywords

Antioxidants, bioactive compounds, gene expression, inflammation, oxidative stress, phytochemicals, transcription factors, vegetables.

PREFACE

Edible plants contain a variety of phytochemicals with biological activities, which act in different ways and interact with different metabolic processes. Notwithstanding, in human nutrition, foods of plant origin are often classified into general categories such as cereals, pulses, fruits, etc. without taking into account the differences exiting among different species or cultivars. These differences account to both macro- and micro-components, and influence the nutritional value of different plant foods and in their impact on health preservation. Particularly, components generally classified as bioactives may vary greatly among different species and cultivars.

The term "bioactive food component" refers to nonessential biomolecules that are present in foods and exhibit the capacity to modulate one or more metabolic processes, which results in the promotion of better health.

Important factor influencing bioactive content in plant-based foods is processing, particularly cooking and freezing. The changes during postharvest handling, storage conditions, processing, and preparation can be beneficial or detrimental to the total content of health-promoting phytochemicals.

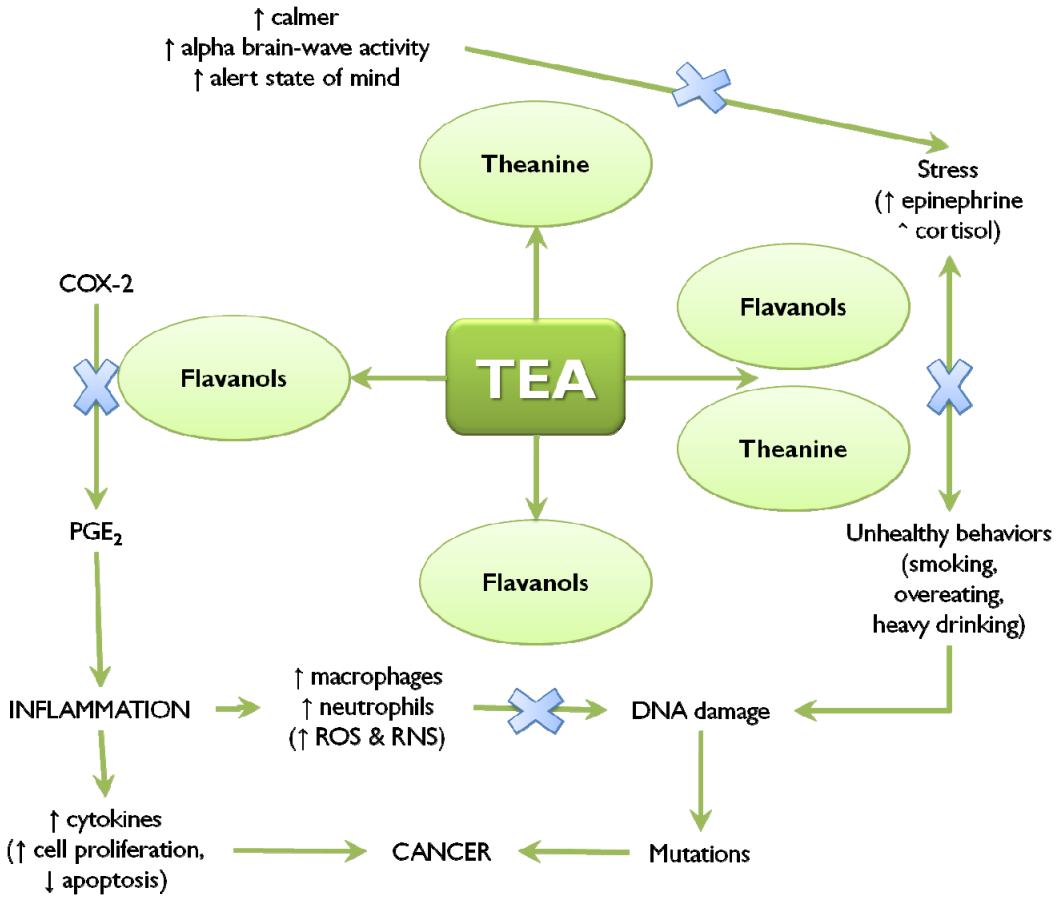
A number of factors, including genetics and growing conditions (that is, fertilization, moisture, pest, and disease burden, and so on), are known to affect the concentration of plant secondary metabolites. The variability in the composition of bioactive molecules, and consequently in the biological activity, of different plant species and cultivars, in relation to environmental and agronomic factors has been seldom investigated. However, existing data are principally related to *in vitro* determinations and not to biological systems. On the contrary, it would be important to demonstrate phytochemical activity in biological systems before giving dietary indications. The nutritional characterization of different edible plants, and their classification on the basis of biological activities, such as antioxidant or anti-inflammatory, could open the way to further researches evidencing the possibility for traditional crops of bearing nutritional and health claims.

Bioactives have attracted great deal of interest because of their perceived ability to act as highly effective antioxidant agents. Pro-oxidative and pro-inflammatory process are pathophysiological factors in the cause of a vast variety of human disorders. Thus, oxidative stress and inflammatory damage are underlying factors implicated in cancer, neurodegenerative disorders, diabetes, etc. as well as aging. In this context, dietary modulation of the cellular antioxidant and anti-inflammatory capacity represents and important strategy to cope with oxidative stress and/or inflammation-mediated pathophysiological disorders.

However, accumulating evidence indicates that phytochemicals exhibit several additional properties in complex biological systems, which may be independent of conventional biological activities. Such novel mechanisms of action include the possibility of influencing gene expression, with consequent modulation of several cell activities. An example of complex interactions between vegetable food and health is provided by green tea, the most commonly consumed beverage around the world. Tea and tea components have a wide range of beneficial healthy effects, such as antioxidant, anti-inflammatory, and chemopreventive. A simplified diagram of the suggested roles of tea components on reduction of inflammation, cancer and improved behavior is represented in **figure 1**.

In conclusion, bioactive food components will play an important role in health maintenance in the future. However, information are needed in regards to the real biological effects of bioactive food components in health promotion and disease prevention.

Figure 1. A simplified diagram of the suggested roles of tea components on reduction of inflammation, cancer and improved behavior. Tea flavanols have anti-inflammatory activity, inhibit COX-2 and iNOS expression by blocking NF-κB activation, and act as blocking and suppressing agents of multistage carcinogenesis. Tea catechins act at multiple brain targets to prevent or delay neuronal death and theanine has been related to improve attention processing and reduce stress.



(Abbreviations used: COX-2: cyclooxygenase-2; iNOS: inductible nitric oxide synthase; NF-κB: nuclear factor κB; PGE_2: prostaglandin E2; RNS: reactive nitrogen species; ROS: reactive oxygen species) (de Mejia E.G., Ramirez-Mares M.V., and Puangpraphant S. *Bioactive components of tea: cancer, inflammation and behavior*. Brain Behav Immun 2009).

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Effect of domestic freezing and cooking on antioxidant activity of edible vegetables

Danesi F., Bordoni A. ***Effect of home freezing and Italian style of cooking on antioxidant activity of edible vegetables****. J Food Sci. 2008; 73(6):H109-H112.*

Abstract

In this study, we analyzed the modifications of antioxidant activity consequent to 3 typical home cooking practices (steaming, boiling, and microwave cooking) in fresh and home frozen vegetables. Six different vegetable species were examined: carrots (Daucus carota L.), zucchini (Cucurbita pepo L.), tomatoes (Solanum lycopersicum L.), green beans (Phaseolus vulgaris L.), peas (Pisum sativum L.), and yellow peppers (Capsicum annuum L.). All vegetables were conventional products and were analyzed in season to minimize differences due to agricultural practice and storage. Cooking and freezing are generally regarded as destructive to antioxidants, and this has fostered a belief among many consumers that raw vegetables are nutritionally superior to their frozen and/or cooked forms. In the current study, we provide evidence that this is not always the case.

Abbreviation used*: AA: antioxidant activity; ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); PBS: phosphate buffered saline; TAA: total antioxidant activity; TE: Trolox equivalent.*

Introduction

Phytochemicals in fruits and vegetables have been receiving increased interest from consumers and researchers for their beneficial health effects on human diseases, mainly due to their antioxidant activity. Epidemiological studies have provided evidence of an inverse association between a vegetable-rich diet and diseases such as cardiovascular diseases[1, 2] and cancer[3]; however, clinical trials using "nutritional" antioxidants as vitamin C and E have given equivocal results[4, 5]. A possible explanation is the presence of other components in foods having higher activity than the well-known nutrient antioxidants. Furthermore, since there are thousands of antioxidants in most foods, the total antioxidant capacity of a given food may result from the integrated and synergistic action of different compounds instead of the sum of each single compound. The concept of total antioxidant activity (TAA) reflects the integrated and, if any, synergic effects of all the antioxidants. It is recognized that foods with high TAA might be protective, and recommendations about a "healthy" diet regard consumption of high amounts of fruit and vegetables.

A number of factors, including genetics and growing conditions (that is, fertilization, moisture, pest, and disease burden, and so on), are known to affect the concentration of plant secondary metabolites having antioxidant activity, therefore modulating their TAA[6]. Processing, particularly cooking of food, is another factor that can impact TAA[7]. It is particularly important for vegetables, since some of them are commonly eaten in cooked form. Thus cooking, especially home cooking, is an important issue that needs to be considered in estimating the daily antioxidant intake. Another important topic about antioxidant activity of vegetables is freezing. Although it is

known that the nutritional quality of the vegetables depends not only on the nutrient content when harvested but also on the changes occurring during postharvest handling, storage conditions, processing, and preparation[8, 9], few studies have quantified the supposed decrease in TAA of vegetables after freezing and cooking at home.

It is a conventional wisdom that processed fruits and vegetables have a lower nutritional values than their respective fresh commodities, mainly due to the loss of vitamin C content in the processing[10]. Knowledge about the effective loss of TAA consequent to home processing may have a significant impact on consumers' food selection and processing.

In this study, we measured the antioxidant activity (AA) related to water soluble antioxidants of 6 different vegetables and compared the results obtained in raw foods to those obtained in cooked (boiled, steamed, and microwave cooked), frozen, and frozen-cooked foods. Vegetables were not thawed before cooking, since thawing significantly reduces the antioxidant components[9].

Materials and Methods

Six different vegetable species were examined: carrots (*Daucus carota* L.), zucchini (*Cucurbita pepo* L.), tomatoes (*Solanum lycopersicum* L.), green beans (*Phaseolus vulgaris* L.), peas (*Pisum sativum* L.), and yellow peppers (*Capsicum annuum* L.).

All samples were bought in season from a local shop the same day of the analysis. Vegetables were weighed, washed, and dried, and possible petioles were eliminated. Each vegetable sample was divided in 2 parts; the first one was immediately used for the analysis on fresh products, the other was frozen in plastic bags, without blanching, at –18 °C in a conventional home freezer (Electrolux, Stockholm, Sweden). Storage lasted for 6 mo at –18 °C.

Fresh vegetables

Vegetables were homogenized in a food processor without addition of any solvent, and 20 g of the homogenized material were dissolved in 60 mL of 5 mM phosphate buffered saline, pH 7.4 (PBS), getting a dilution 1:3 (w/v). Yellow pepper (fresh and cooked) samples were diluted 1:10 (w/v), due to their high antioxidant activity. The samples were then filtered and the resulting filtrate was used to determine the antioxidant activity (AA). All phases of homogenization were done in ice and each extract immediately analyzed. Two replicates were prepared from each sample, and at least 3 different samples, bought in different days, were considered for each vegetable.

Frozen vegetables

Before analysis, frozen vegetables were weighed again to evaluate possible variation induced by freezing, and then they were directly processed as described for fresh vegetables without defrosting.

Cooked vegetables

Vegetables were cooked using 3 different home processing methods: boiling (20 min in 1.5 L of salty water); steaming (30 min); and microwave (700 W for 5 min, followed by a standing for 2 min). Vegetables were cooked as a whole, without cutting, peeling, and eventually defrosting, and then processed as described for fresh samples. For each sample, the amount of homogenate dissolved in 60 mL PBS was calculated taking into account possible weight variation due to freezing or cooking, and corresponded to 20 g of the fresh, uncooked vegetable.

Antioxidant activity (AA)

AA was measured using the method of Re *et al.*[11] on the basis of the ability of the antioxidant molecules in the sample to reduce the radical cation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), determined by the decolorization of $ABTS^{+\cdot}$, and measured as the

quenching of the absorbance at 734 nm. Values obtained for each sample were compared to the concentration–response curve of the standard trolox solution and expressed as micromoles of Trolox equivalent (TE).

All data are means ± SD. Statistical analyses were carried out using the Student's *t*-test comparing value of fresh cooked vegetables and frozen cooked vegetables with the corresponding fresh uncooked one, and by the 1-way analysis of variance (ANOVA) followed by Bonferroni test to compare the 3 cooking methods for each fresh and frozen vegetable.

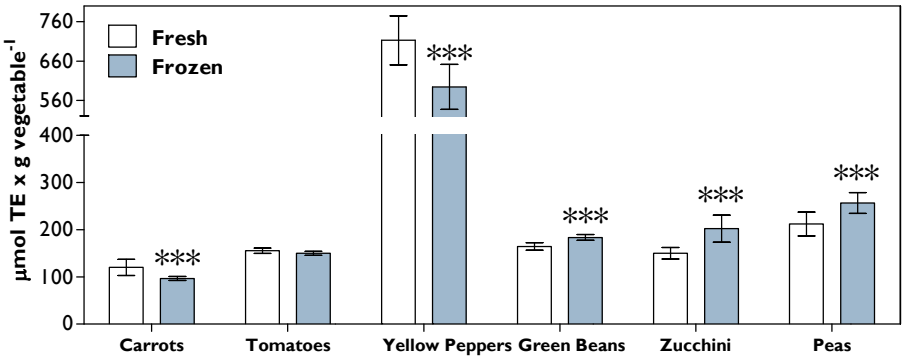
Results and Discussion

Although antioxidants in plant can be divided in lipophilic, hydrophilic, and amphiphatic compounds, and antioxidant activity may be accounted to all components, we decided to assess only the modification of antioxidant activity due to hydrophilic/amphiphatic ones (AA) for 2 reasons: (1) the contribution to total antioxidant activity of lipophilic antioxidant in the examined vegetables is very poor[12]; (2) evaluation of the activity of lipophilic antioxidants requires the use of different solvents, causing an extraction of lipophilic components that could be much more exhaustive than the digestive process, so causing nonphysiological results.

Our results in uncooked foods indicate not only that AA varies in different fresh vegetables according to their qualitative/quantitative content of antioxidants, but also that the effect of home freezing largely depends on the type of vegetables (**Figure 1**). Notably, freezing increased AA in green vegetables (green beans, zucchini, and peas), while decreasing or not affecting it in yellow/red ones (carrots, tomatoes, and yellow pepper). Phenolic compounds are associated not only with AA, but also with color characteristic of vegetables; it is conceivable that compounds related to green color are more resistant to freezing than those related to yellow/red.

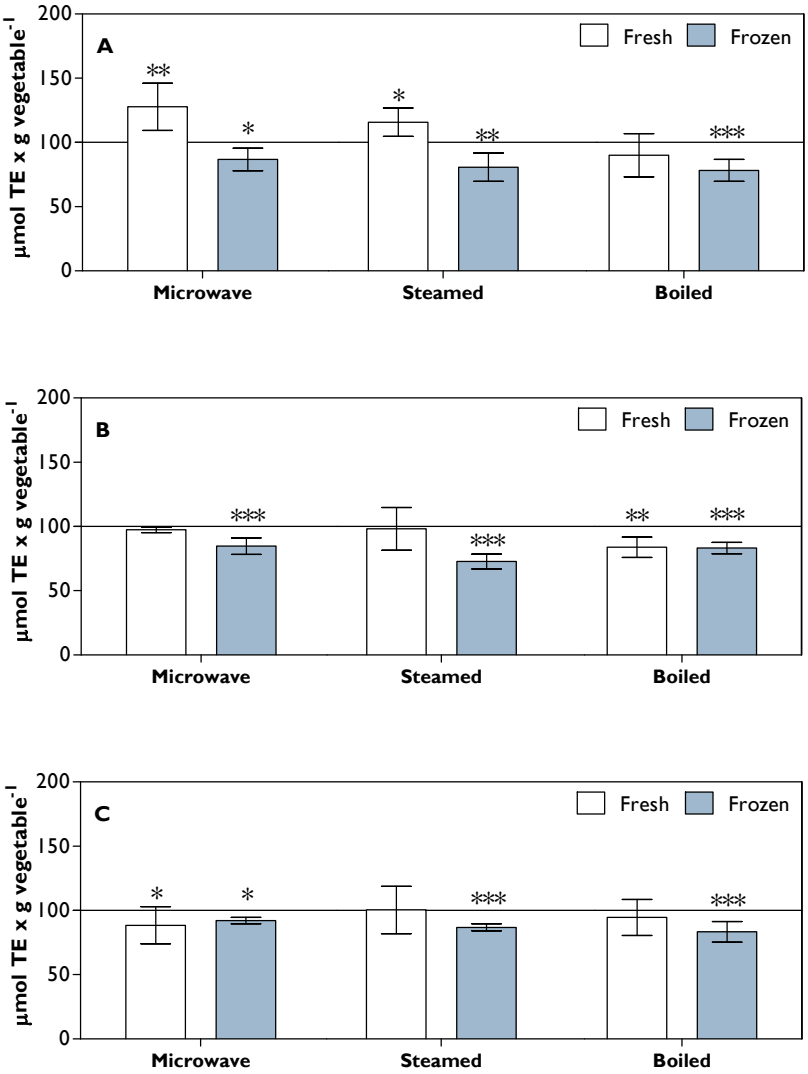
The cooking practices differently influenced the AA with respect to raw material, depending on the vegetable considered and the storage condition (fresh or frozen). Vegetables were not peeled before cooking or freezing, since numerous phenolic compounds are located in the periderm of vegetables[13]; it has been demonstrated that in onions the greatest loss of flavonoids takes place during the preprocessing step, where the onion is peeled, trimmed, and chopped[14].

Figure 1. Antioxidant activity (AA) in fresh and frozen vegetables. AA was determined as reported in Methods, and is expressed in µmol Trolox equivalent (TE) per gram vegetable.



*Data are means ± SD of 6 different samples in each condition. Statistical analysis was done by the Student's t-test comparing frozen and the corresponding fresh vegetable: ***P < 0.001.*

Figure 2. Modification of AA in fresh and frozen yellow/red vegetables after cooking. AA was determined as reported in Methods, and is expressed in µmol Trolox equivalent (TE) per gram vegetable. AA of fresh uncooked vegetables was set as 100, and modifications due to cooking are indicated as increase or decrease with respect to 100. Data refer to carrots (A), red tomatoes (B), and yellow peppers (C), and are means ± SD of 6 samples in each condition.



*Statistical analysis was done by the Student's t-test comparing value of fresh cooked vegetables and frozen cooked vegetables with the corresponding fresh uncooked one (*P < 0.05; **P < 0.01; ***P < 0.001) and by the 1-way ANOVA followed by Bonferroni test comparing the 3 cooking methods for each fresh and frozen vegetable (carrots, fresh P < 0.001; red tomatoes, frozen P < 0.01; yellow pepper, frozen P < 0.01).*

Regarding orange/red vegetables, AA of fresh carrots was increased or unaffected by cooking (Figure 2A), in agreement with the data of Talcott and Howard[15], who observed a 70%

increase of carrot total soluble phenolics immediately after thermal processing. The increase in AA due to thermal process has been mainly attributed to transformation of chlorogenic acid, the predominate phenolic acid in carrots, into caffeic acid[16]. Statistical analysis indicated boiling as the worst cooking practice in fresh carrots. Since phenolic antioxidants are water soluble and can be leached from vegetable tissues by processing in water, this can explain the highest AA decrease after boiling. In frozen carrots, AA was significantly decreased by cooking; it is conceivable that freezing had altered the peel barrier, allowing a loss of antioxidants during the cooking procedures.

AA in fresh tomatoes was decreased by boiling, while in frozen tomatoes it was significantly affected by all cooking procedures (**Figure 2B**). Tomato is widely consumed either fresh or after processing into various (cooked) products, and its consumption has been proposed to reduce the risk of several chronic diseases. These health protective effects have been widely attributed to the presence of key antioxidants such as lipid-soluble lycopene and β-carotene as well as water-soluble vitamin C, and compounds of intermediate hydrophobicity such as quercetin glycosides, naringenin chalcone, and chlorogenic acid. All of these are known to contribute significantly to the antioxidant activity of tomato[17]. In the literature, there are several reports on the effects of processing on tomato compounds, or on the total antioxidant potential of the processed tomato materials, and a number of studies indicate that the content of carotenoids and vitamin C may be negatively affected by various thermal treatments such as boiling, frying, drying, and microwaving[18]. Little is known about the effects of thermal treatments on tomato (poly)phenolic antioxidants, including flavonoids. Since in this study we measured only AA related to hydrophilic compounds, our results indicate a loss of water-soluble molecules due to cooking. This loss was higher in frozen tomatoes, possibly due to the alteration of the peel barrier. This decrease in AA is in agreement with the recent study of Capanoglu *et al.*[19], who showed that processing significantly affects the levels of antioxidants and many other metabolites present in commercial tomato paste.

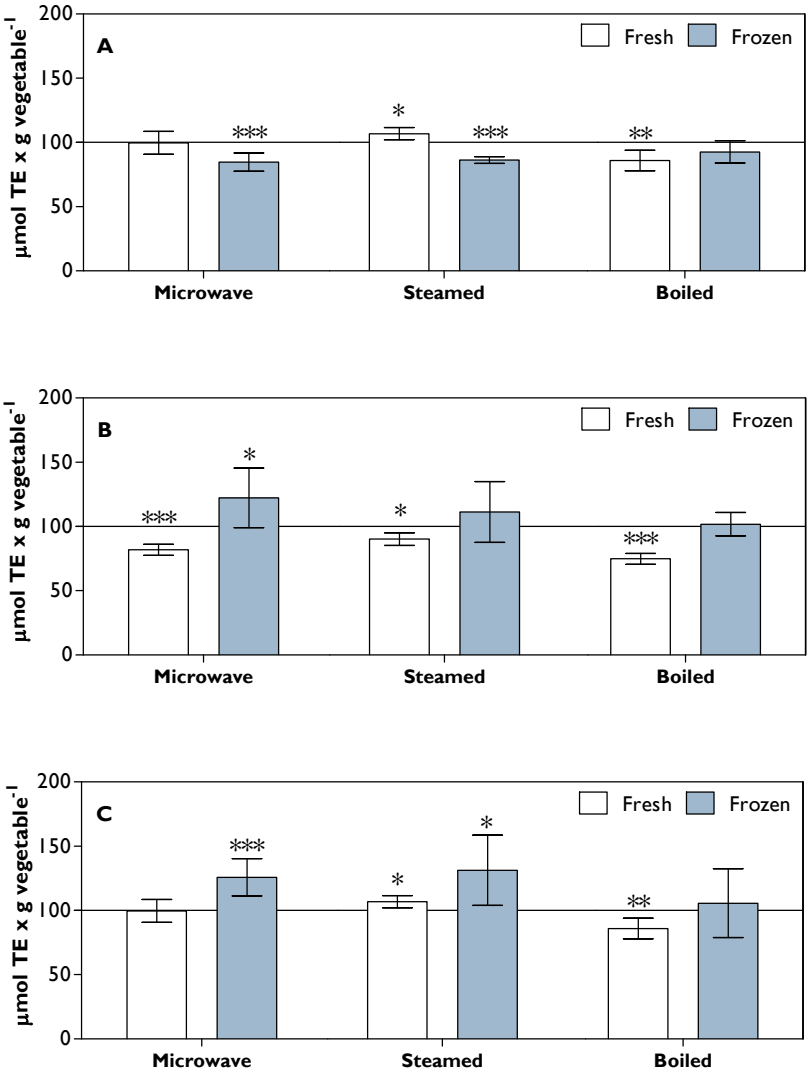
AA was decreased by microwave cooking in fresh yellow pepper and by all cooking practices in frozen *Capsicum annul* (**Figure 2C**), allowing hypothesizing that both freezing and high frequency may alter the barrier effect of peel, therefore causing antioxidant loss. The higher decrease in AA in frozen yellow peppers after cooking could be related to the reported decrease in ascorbic acid content due to the exposure to low temperature[20].

Among green vegetables, AA of fresh green beans was reduced by boiling and increased by steaming (**Figure 3A**), in agreement with Masrizal *et al.*[21], who observed that boiling of green beans causes the major loss of vitamin C. Steaming and microwave cooking reduced AA in frozen green beans; this decrease could be mainly attributed to the loss of ascorbic acid, since green beans do not retain their vitamin C very well. Lee and Kader[22] categorizing different vegetables as being good, moderate, or poor in their retention of ascorbic acid, and designated green beans as poor.

Fresh zucchini appeared very sensitive to cooking, their AA decreasing independently from the practice used. Conversely, AA of frozen zucchini did not decrease after cooking, with microwave practice actually increasing it (**Figure 3B**). Little data are available in the literature on cooked zucchini, but the negative effect of boiling is in agreement with Andlauer *et al.*.

Finally, AA of fresh peas was modified by boiling only (**Figure 3C**), probably due to the least percentage loss of vitamin C in these vegetables compared to others[24]. Accordingly to Hunter and Fletcher, who showed, after a 20% loss during blanching, no further loss of ascorbic acid during freezing of peas, frozen peas appeared particularly resistant to AA loss due to cooking (**Figure 3C**).

Figure 3. Modification of AA in fresh and frozen green vegetables after cooking. AA was determined as reported in Methods, and is expressed in µmol Trolox equivalent (TE) per gram vegetable. AA of fresh uncooked vegetables was set as 100, and modifications due to cooking are indicated as increase or decrease with respect to 100. Data refer to green beans (A), zucchini (B), and peas (C), and are means ± SD of 6 samples in each condition.



*Statistical analysis was done the Student's t-test comparing value of fresh cooked vegetables and frozen cooked vegetables with the corresponding fresh uncooked one (*P < 0.05; **P < 0.01; ***P < 0.001) and by the 1-way ANOVA followed by Bonferroni test comparing the 3 cooking methods for each fresh and frozen vegetable (green beans, fresh P < 0.001; zucchini, fresh P < 0.001; peas, fresh P < 0.001).*

Our results partially agree with those of Ewald *et al.*[14], who reported small effects on flavonoid content due to cooking of green beans and peas.

The antioxidant profile has been recognized to represent an important parameter to predict the impact of food on human health, and a growing body of evidence suggests that compounds with antioxidant activity play a major role in explaining the benefits of vegetable consumption.

Cooking and freezing are generally regarded as destructive to antioxidants, so supporting the consumers' assumption that only fresh vegetables are healthy. It is documented that vitamin C is lost during processing, but ascorbic acid represents only a minor part of the AA, polyphenols being very important antioxidant compounds even in vegetables where the vitamin is present in high concentration[26]. Notwithstanding, there are limited data on polyphenol content, and even less data on AA, of cooked vegetables. Information on AA in terms of the food as eaten needs to be generated, taking into account that home cooking still represents the most frequent method of food processing, and it is applied in different ways not only to fresh foods but also to frozen ones. One of the main differences between home freezing and industrial deep-freezing is the blanching procedure, which is generally not used in home practice. Studies performed on deep-frozen vegetables do not allow us to understand the impact of low temperatures on antioxidant activity of vegetables, since blanching often modifies it much more than freezing[25, 27].

In this study, we have shown that the assumption that frozen vegetables have a lower antioxidant potential than fresh ones is not a universal truth, but depends on the vegetable considered. Cooking negatively affects green vegetables AA more than freezing, and frozen cooked vegetables often present a higher AA than the corresponding fresh ones. Conversely, frozen yellow/red vegetables are more susceptible to AA loss due to cooking than the corresponding fresh ones. This has to be carefully considered since frozen vegetables are always cooked before consumption. Regarding cooking practice, boiling often appears to be the worst practice in terms of AA preservation.

Overall, changes in AA appeared dependent on the type of vegetable considered, as already reported by Turkmen *et al.*[28], and on the type of cooking practice.

Conclusions

Nowadays, consumers are aware of the need to consume a variety of fresh vegetables to get the most complete antioxidant support[29]; however, many people do not have the opportunity to eat fresh vegetables every day and frequently use frozen vegetables, and many vegetables are consumed as cooked. The aim of this study was neither a classification of different plant foods on the basis of their total antioxidant activity nor the analysis of the modifications occurring in single antioxidant molecules after thermal treatment, but the evaluation of AA modification occurring in the same sample of vegetable food after freezing, cooking, and freezing/cooking. Our results indicate that, at least for hydrophilic molecules, changes in AA due to home processing are very different in different vegetables. This is probably related to their different contents of vitamins, carotenoids, and phenolic compounds, which act synergistically but are differently sensitive to processing. Overall, the decrease in AA occurring after home processing is relatively small, and it is not likely to cause a significant decrease in antioxidant intake, at least in the considered conditions. Notwithstanding, it remains that home processing is an important issue that needs to be considered in estimating the daily total antioxidant intake, and better measurements and understanding of AA on common foods may help nutritionists.

Acknowledgments

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Influence of agronomic techniques on the antioxidant activity of aqueous extracts from palm tree kale in protecting cardiomyocytes from oxidative stress

Danesi F., Di Nunzio M., Elementi S., Neri R., D'Antuono L.F., Bordoni A. ***Effectiveness of essential oils and extracts derived from edible plants in protecting cardiomyocytes from oxidative stress. Influence of cultivar and agronomic techniques.*** *2° Verona International Meeting on Foods, Phytotherapic Compounds and Health, Verona (VR) 29-30 settembre 2007.*

Abstract

The present study evaluates the biological effects of extracts derived from a kale cultivar, Palm Tree Kale (Brassica oleracea L., ssp. acephala Dc, var. sabellica L.), cultivated with different agronomic techniques. The study was performed using primary cultures of neonatal rat cardiomyocytes which were grown in media supplemented with different concentrations of the extracts. At confluence cells were submitted to an oxidative stress, in order to evaluate the protective effects of the different supplementations. The final aim of the study was to evaluate the biological activity of Palm Tree Kale extracts, comparing it to the activity of a well-known antioxidant, and to verify if differences in extract composition, as determined by agronomic techniques, may affect their biological activities. The significant differences observed, related not only to the concentration of the supplemented extracts but also to the agronomic techniques used in growing Palm Tree Kale, indicate the importance of the selection of raw material as source of bioactive compounds.

Introduction

It is reported that diets rich in fruits and vegetables are protective against chronic diseases such as cancer and heart diseases[1-3]. These protective effects are generally attributed to the presence of various functional components, such as phenolic compounds, vitamin C, vitamin E, provitamins, minerals, and fiber. Many of these compounds have bioactive mechanisms for effectively scavenging reactive oxygen species (ROS) and reducing cell proliferation in cancer cell lines[4, 5]. Oxidative stress appears to be the critical factor in the pathogenesis of many diseases because ROS have the ability to damage macromolecules like DNA, protein, and lipids[6, 7]. To minimize the harmful effects of oxidative stress

Abbreviation used: *ABTS: 2,2'-azinobis(3-ethylbenzo-6-thiazoline-6-sulfonic acid); DNA: deoxyribonucleic acid; EBSS: Earl's balanced salt solution; FCS: fetal calf serum; HS: horse serum; LDH: lactate dehydrogenase; MTT: 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium-bromid; NADH: nicotinamide adenine dinucleotide (reduced form); ROS: reactive oxygen species; TAA: total antioxidant activity; TC: α-tocopherol; TE: Trolox equivalent; UV: ultraviolet; WTO: World Trade Organization.*

in the human body, it is necessary to supply adequate amounts of ROS-scavengers, and fruits and vegetables are considered to be the major contributors of ROS-scavenging antioxidants.

Different vegetables contain different bioactive compounds exerting different effects; in this regard, *Brassicacea*e are of particular interest as they are characterised by the presence of glucosinolates, organic sulphur compounds that, besides determining the organoleptic characteristics of food species, also possess high biological activity, such as antioxidant and antitumoral activity[8, 9].

Nutrient composition of vegetables is very complex and difficult to assess. Levels of plant metabolites are strongly affected by genetic and environmental factors as well as transportation and storage conditions. Growth factors such as light, temperature, humidity, type of soil, application of fertilizers, damage caused by microorganisms and insects, stress induced by UV radiation, heavy metals, and pesticides all alter metabolite composition of plants[10]. In the literature indeed, the variability in the composition of bioactive molecules, and consequently in the biological activity, of *Brassicaceae* species in relation to agronomic factors has been seldom investigated and existing data are principally related to *in vitro* determinations and not to biological systems. Tests in biological systems are, however, necessary to confirm their real effectiveness as protecting agents.

Therefore, the main objective of this research was to verify the protective effect of extract derived from plants of the same species and cultivar but grown at two plant density (traditional and high), and following different agronomic techniques (two different doses of nitrogen fertilization: 0 and 160 kg x ha^{-1}).

Soil mineral nitrogen availability may vary according to the amount of applied nitrogen and crop nitrogen uptake pattern[11]. Nitrogen uptake, which is genetically determined, in *Brassica* is higher than in many other food crops. Despite this, a significant part of the nitrogen taken up is lost to the soil in dead leaves, during the growth cycle[12]. In contrast to conventional systems, in which synthetic fertilizers containing directly available inorganic nitrogen are used, organic systems rely on the activity of a diverse soil ecosystem to make nitrogen available to plants[13, 14]. It has been shown that nitrogen can become a growth-limiting nutrient in organic production, affecting negatively yield[14].

In this study we verified the potential protective effects of the different hydrosoluble extracts of Palm Tree Kale, with particular attention to their possibility to counteract the oxidative damage due to ROS. We used primary cultures of neonatal rat cardiomyocytes as model system. The heart is an aerobic organ, and most of the energy required for the contraction and maintenance of ion gradients comes from oxidative phosphorylation. Oxidative stress caused by free radicals plays a crucial role in the pathophysiology associated with cardiovascular diseases; therefore, a great deal of attention has focused on the naturally occurring antioxidant phytochemicals as potential therapy.

Aqueous extracts of Palm Tree Kale were supplemented to cultured cardiomyocytes, and their protective effect was evaluated in both normal conditions and after an oxidative stress, comparing it to the effect of 20 μM α-tocopherol (TC) supplementation. TC is considered to be the most important endogenous antioxidant in cardiac cells[12], and it is often used as positive control in studies regarding antioxidant protection in cultured cardiomyocytes.

The final aim of the study was to evaluate the biological activity of Palm Tree Kale extracts, comparing it to the activity of a well-known

antioxidant, and to verify if differences in extract composition, as determined by agronomic techniques, may affect their biological activities.

Materials and methods

Materials

Horse serum (HS), fetal calf serum (FCS), Ham F10 culture medium, 2,2′-azinobis(3-ethylbenzo-6-thiazoline-6-sulfonic acid) (ABTS), Trolox, α-tocopherol, and other biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). α-Tocopherol (TC) was dissolved in ethanol at a concentration of 5 mM and kept at −20 °C until use. All of the chemicals and solvents were of the highest analytical grade.

Palm Tree Kale seeds were obtained by a local seed company based in Cesena and directly sown in the open field on March 21, 2006, in the Cadriano experimental farm, near Bologna. A base fertilization of 80 kg ha^{-1} P_2O_5 and 50 K_2O was applied, pre-sowing. No phytochemicals or irrigation were applied. The experimental theses were:

A. two planting densities: 1 plant m^{-2}, corresponding to the plant population of traditional, handpicked, crops; 240 plants m^{-2}, corresponding to the density adopted in mechanized industrial crops;

B. nitrogen fertilization in both density plots: a dose of 160 kg ha^{-1} nitrogen; a higher density plot was unfertilised control (table 1).

Table 1. Agronomic techniques used in different theses of Palm Tree Kale.

type	plant density	nitrogen fertilization
HD-0N	240 plant m^{-2}	0 kg N ha^{-1}
HD-160N	240 plant m^{-2}	160 kg N ha^{-1}
TD-160N	1 plant m^{-2}	160 kg N ha^{-1}

The plots were arranged in a randomised block experimental design with three replications. Mature leaves were harvested on June 21 2006.

Methods

Aqueous Extracts Preparation

Twenty g of lyophilized Palm Tree Kale leaves, ground to a fine powder in a mill, were mixed by magnetic stirrer with 500 mL of boiling water and then filtered over Whatman no. 1 paper. The filtrates were frozen and lyophilized in a lyophilizer at 5 mmHg pressure and at −50 °C and then redissolved to solubilize the amount of lyophilized extract derived from 100 mg of fresh leaves in 1 mL of sterile bidistilled water. The obtained solutions were filtered on 0.20 μM filters (Whatman Europe, U.K.) and then used for cardiomyocytes supplementation.

Aqueous Extracts Analysis

Total phenol content of the aqueous extracts was determined according to Gao *et al.*[15], with slight modifications: 100 mg of lyophilized material was extracted with 0.99 mL of 80% (v/v) ethanol + 0.01 mL of 36% (v/v) HCl.

Total Antioxidant Activity (TAA) of Extracts

TAA was measured using the method of Re *et al.*[16], based on the capacity of antioxidant molecules to reduce the radical cation of ABTS, determined by the decoloration of $ABTS^{\bullet+}$, and measured as the quenching of the absorbance at 734 nm. Values obtained for each sample were compared to the concentration−response curve of the standard Trolox solution and expressed as micromoles of Trolox equivalent (TE).

Cell Cultures

Primary cultures of cardiomyocytes were obtained from the ventricles of newborn Wistar rats according to the method of Yagev *et al.*[17].

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication 85-23, revised 1996). To obtain pure cultures avoiding the presence of fibroblasts, cells were preplated twice before the final seeding, and the absence of cells other than cardiomyocytes was verified microscopically. Cells were randomly divided in the control, grown in Ham F10 medium plus 10% fetal calf serum plus 10% horse serum, and supplemented cardiomyocytes, grown in the same medium but supplemented with different concentrations of extract solutions (10 or 20 µL mL^{-1} medium), or with 20 µM TC. Media were changed every 48 h, the last medium change being 48 h before the experiments. At complete confluence in a monolayer, media were removed and substituted with Earl's Balanced Salt Solution (EBSS), pH 7.4 (116 mM NaCl, 5.4 mM KCl, 0.9 mM $NaH_2PO_4 \cdot 2H_2O$, 26 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4 \cdot 7H_2O$).

To cause an oxidative stress, some cells received EBSS buffer added with 0.1 mM hydrogen peroxide. After 1 h, EBSS buffer was removed and collected for the determination of lactate dehydrogenase activity; cardiomyocytes were washed three times with 0.9% NaCl, and cell proliferative activity was assayed.

Lactate Dehydrogenase (LDH) Activity in Media

Cardiomyocyte plasma membrane integrity was assessed by measuring the release of LDH to the EBSS buffer. Enzyme activity was determined spectrophotometrically by measuring NADH levels at 340 nm[18].

Cell Proliferative Activity

Cell proliferative activity was measured using the 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay[19]. The test is based on the capacity of mitochondrial dehydrogenase in viable cells to convert MTT reagent to a soluble blue formazan dye. Briefly, after a washing with 0.9% NaCl, 1 mL of MTT reagent diluted in RPMI-1640 medium modified without phenol red (1 mg mL^{-1}) was added to each dish, and the cell cultures were incubated for 3 h at 37°C. After removal of the medium, the cells were lysed with isopropanol for 15−20 min. Formazan production, which is proportional to cell vitality, was determined spectrophotometrically at 560 nm.

Statistical Analysis

The reported data are the means of at least three samples obtained from different cell cultures. Statistical analysis was carried out by means of the one-way and two-way ANOVA, and by the Students' t test.

Results

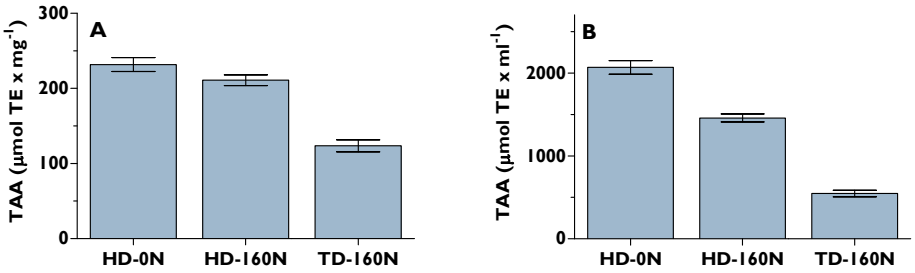
The total phenolic content of the different aqueous extracts is reported in table 2. HD-0N and HD-160N extracts appeared almost similar, while TD-160N extract revealed a lower phenolic content ($p<0.001$).

Table 2. Total phenolic content (mg GAE g^{-1} lyophilised extract) of Palm Tree Kale lyophilized aqueous extracts.

type	total phenolic content
HD-0N	30,45 ± 0,02
HD-160N	29,68 ± 0,13
TD-160N	19,25 ± 0,51

Data are mean ± SD of 3 samples in each group. Statistical analysis was by one way Anova: p<0.001.

Figure 1. Total antioxidant activity (TAA) of Palm Tree Kale aqueous extracts.

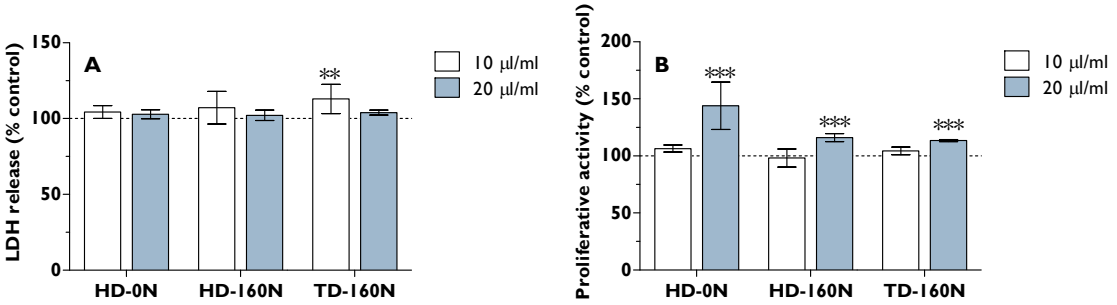


TAA is reported as µmol TE x mg^{-1} (A) and µmol TE x ml^{-1} (B). Data are means ± SD of three replicates. Statistical analysis was by one way ANOVA: p<0.001 in both cases.

Total antioxidant activity was calculated as micromol trolox equivalent (TE) x mg^{-1} extract (figure 1A) and x ml^{-1} solubilized extract (figure 1B). In the first case, according to the lower phenolic content, the *in vitro* TAA of TD-160N extract was lower than the other two. In the second case, TAA of HD-160N extract appeared lower than HD-0N one.

In basal condition, supplementation of cardiomyocytes with Palm Tree Kale aqueous extracts did not cause modification in LDH release with respect to controls, apart from a slight increase due to TD-160N extract at the lowest dose (figure 2A). Proliferative activity was not modified by the supplementation with the lowest concentration of all extracts. Conversely, at the highest dose used a significant increase in cell proliferative activity was detected in comparison to controls, regardless the type of extract used (figure 2B).

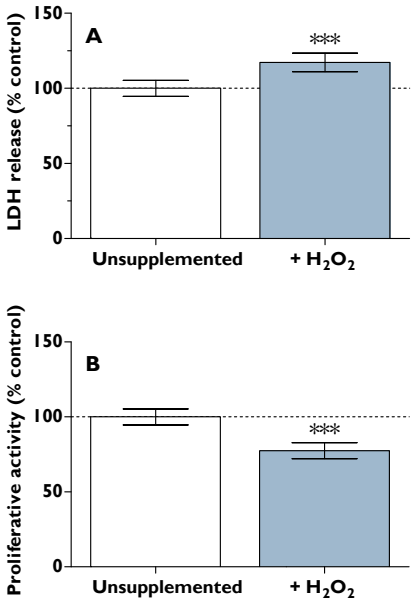
Figure 2. LDH release (A) and proliferative activity (B) in cardiomyocytes supplemented with Palm Tree Kale aqueous extracts.



*Cardiomyocytes were supplemented with Palm Tree Kale aqueous extracts at two concentrations: 10 and 20 µl ml^{-1} medium. Data are expressed as percent of value in control cells. Statistical analysis was by one day ANOVA among the different extract (10 µl/ml LDH n.s. and MTT n.s.; 20 µl/ml LDH n.s. and MTT p<0.05), and by the Student's t-test in comparison to control cells (** p<0.01; *** p<0.001).*

The addition of H_2O_2 to the medium of cardiomyocytes appeared to be cytotoxic, causing a significant increase in LDH release and a significant decrease in cell proliferative activity with respect to basal condition (**figure 3A** and **B**).

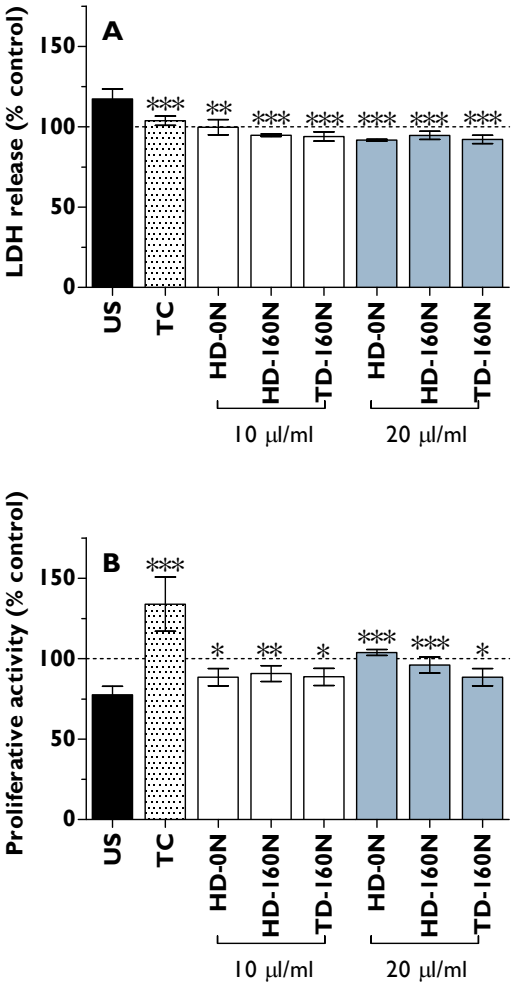
Figure 3. LDH release (A) and proliferative activity (B) in stressed cardiomyocytes unsupplemented.



*Data are expressed as percent of value in non-stressed unsupplemented cells. Statistical analysis was by the Student's t-test in comparison to non-stressed unsupplemented cells (*** p<0.001).*

The increase of LDH release was totally prevented by the supplementation with TC and with Palm Tree Kale extracts at both concentrations. In some cases, LDH release in cells supplemented with Palm Tree Kale extracts was even lower than in control cells in basal condition (**figure 4A**).

Figure 4. LDH release (A) and proliferative activity (B) in stressed cardiomyocytes supplemented with tocopherol or Palm Tree Kale aqueous extracts.



Cardiomyocytes were supplemented with Palm Tree Kale aqueous extracts at 10 or 20 µl ml^{-1} medium concentration. Data are expressed as percent of value in non-stressed unsupplemented cells. Statistical analysis was by one day ANOVA among the different extract (10 µl/ml LDH n.s. and MTT n.s.; 20 µl/ml LDH n.s. and MTT p<0.05), and by the Student's t-test in comparison to unsupplemented, stressed cells (p<0.05; ** p<0.01; *** p<0.001).*

The decrease in proliferative activity was totally prevented by TC supplementation. Palm Tree Kale extracts also sustained proliferative activity, although to different extent. After supplementation at 10 μg/ml, proliferative activity was higher in cells supplemented with HD-160N extract; this extract was the most efficient, together with HD-0N extract, when supplemented at 20 μg/ml concentration (**figure 4B**).

Discussion

Results obtained show that, in basal condition, supplementation of cultured cardiomyocytes with different soluble extracts derived from Palm Tree Kale not only had no toxic effect, but also sustained cell vitality. In this light, the most efficient extract was the HD-0N one.

The damage due to exposure to hydrogen peroxide was assessed by the increased release of LDH in the culture medium and by the reduction of cell proliferative activity. Supplementation with Palm Tree Kale extracts counteracted both effects of hydrogen peroxide, protecting cells from oxidative damage. Regarding LDH release, the effect was similar for all extracts at both concentration used, and comparable to the effect of TC supplementation.

TC was supplement at the concentration that is reported as the most effective one in cardiac cells, so it is not surprising that its effect in maintaining cell vitality was higher than for extracts. Notwithstanding, after the oxidative stress proliferative activity was higher in Palm Tree Kale supplemented cells than in unsupplemented ones. This effect appeared clearly dose-dependent, and was lower in TD-160N extract than in the other two.

The lower efficiency in cell protection of TD-160N extract in oxidative condition could be related to the lower content of phenolic compounds. No difference in phenolic content was detected between HD-0N and HD-160N extracts. These two extracts derived from Palm Tree Kale grown at the same plant density (high density), without or with nitrogen fertilization (0 and 160 kg x ha^{-1}, respectively). It has been reported that fertilization with nitrogen usually accelerates the vegetative growth of plants, although

increased nitrogen availability may decrease the levels of phenolic compounds[20]. Although Sousa *et al.*[21] indicate that minimized nitrogen fertilization is necessary to obtain high phenolics content in *Brassica oleracea* var. *costata*, our data indicate that nitrogen fertilization is not a determinant of phenolic content, at least in Palm Tree Kale.

The low impact of nitrogen fertilization on phenolic content is confirmed by the comparison between HD-160N and TD-160N extracts. In fact, notwithstanding the same nitrogen fertilization, these two extracts had different phenolic content. Since HD-160N and TD-160N Palm Tree Kale cultures differed mainly for plant density (high and traditional, respectively), this agronomic parameter seems particularly important in determining phenolic content.

It is interesting to note that TAA of extract reflected phenolic content when expressed per unit weight of extracts. On the contrary, expressing TAA as μmol TE x ml^{-1} of extract solutions we found higher value for HD-0N extract. Since the same solutions were used for supplementing cardiomyocytes, we expected a lower protective effect of HD-160N extract solution than HD-0N one. Since it was not the case, our data confirm that measurement of TAA of foods or extracts in *in vitro* model system does not completely reflect their biological activity, in agreement with Danesi *et al.*[22].

Kale is a leafy green vegetable that belongs to the cabbage family (*Brassicaceae*), a group of vegetables including cabbage, broccoli, cauliflower

and Brussels sprouts that recently have gained increased attention due to their high content of health-promoting phytochemicals. Kale is a leafy vegetable that does not form heads like cabbage or edible flower buds like broccoli and cauliflower. Kale has a high nutritive value due to its richness of phytochemicals, with high concentration of vitamins, minerals, dietary fiber, glucosinolates, and antioxidative compounds, including polyphenols and phenolic acids[23, 24]. It is generally assumed that the beneficial effects of kale and other vegetables are partly attributed to the complex mixture of phytochemicals possessing antioxidant activity[23, 25]. Considering the chemical diversity of the antioxidant compounds present in kale and the interaction occurring among those different molecules, in this study we decided to classify the potential protective effect of different kale extracts by evaluating their total antioxidant capacity, which is a more useful marker than the evaluation of single compound.

In vitro TAA reflected only partially the biological effects of the different extracts, underlying that biological measurements are needed before drawing conclusion on the efficacy of different plant food. Anyway, all extracts appeared protective against an oxidative stress in our model system, their efficacy appearing related to the phenolic content of the extract.

Variation in the polyphenolic content is related to the biosynthesis in the plant, which is influenced by many factors, such as cultivar, climate, postharvest treatments, and agricultural and environmental factors[23]. In order to avoid too many interferences in phenolic content, we decided to test extracts derived from plant of the same cultivar, grown in the same period and in the same place, harvested in the same way and receiving the same postharvest treatment. Only two variables were introduced, i.e. nitrogen fertilization and plant density. The second one appeared to influence phenolic content much more than the first one.

The effect of different factors on the content of bioactive compounds in kale has been poorly studied, and our results could be important in the selection of agronomic techniques able to produce food with high protective activity.

We performed our study on an old variety of kale, i.e. Palm Tree Kale. Particularly, Palm Tree Kale is a local kale type, grown in some Italian areas of inland Tuscany and northern Apennines[26], where it is used as food, both raw and cooked, but, differently from other cabbages, its polyphenol composition has not yet been studied[27-29]. Only Romani *et al.*[30] analysed *Brassica oleracea var. Acephala* Dc. subvar. *Viridis* cv. *Serotina* polyphenols composition in relation to the environment.

Heimler *et al.*[31], in a study examining the content of total phenolics and flavonoids in different *Brassica* plants indicate that "Black Kale" (*Brassica oleracea* L. var. *acephala* Dc), together with broccoli, exhibit the highest content of both classes of bioactives. Those data and our data indicate the important role of kale in human diet for the prevention of oxidative stress, underlying the importance of old, traditional crops.

In the current era, national geographical boundaries of the countries are shrinking and the term "globalization" is an inevitable phenomenon. The World Trade Organization (WTO) encouraged farmers to adjust their cropping structure, and the increased incomes that accompanied the shift of farmers to more profitable crops was accomplished by the disappearance of some traditional crops[32]. Interestingly, some traditional crops survived in different geographical areas, let us hypothesize that they have an intrinsic nutritional value behind their scarce economical appeal.

Although further studies are needed, our data seem to confirm this hypothesis, at least for Palm Tree Kale.

Acknowledgment

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Effect of cultivar on the protection of cardiomyocytes from oxidative stress by essential oils and aqueous extracts of basil

Danesi F., Elementi S., Neri R., Maranesi M., D'Antuono L.F., Bordoni A. ***Effect of cultivar on the protection of cardiomyocytes from oxidative stress by essential oils and aqueous extracts of basil (Ocimum basilicum L.)****. J Agric Food Chem. 2008; 56(21): 9911-9917.*

Abstract

Notwithstanding the wide range of biological and pharmacological activities reported for sweet basil (Ocimum basilicum L.), many discrepancies are still present in the evaluation of its health-promoting properties. These discordances could be at least in part due to insufficient details of qualitative and quantitative composition, connected to the ample variability of this species. Furthermore, many investigations have been carried out in vitro, with few data available on the effectiveness in biological systems. In this study, the protective effect of essential oils and water-soluble extracts derived from three different cultivars of sweet basil has been evaluated in cultured cardiomyocytes. To verify the effectiveness of supplemented oils/extracts in counteracting oxidative damage, cardiomyocytes were stressed by the addition of hydrogen peroxide. The results indicate that (a) in vitro antioxidant activity is not predictive of biological activity and (b) basil can yield extracts with substantially different protective effects, in relation to composition and extraction techniques. Variation among different cultivars has also been detected.

Introduction

Natural antioxidants of plant origin belong to several chemical and functional categories, mostly vitamins, phenolic compounds, including flavonoids and phenolic acids, and volatile compounds. These natural antioxidants are becoming increasingly important not only in the food industry, because they retard oxidative degradation of lipids and thereby improve the quality and nutritive value of foods, but also in medicine, for the prevention and cure of diseases associated with oxidative damage.

Notwithstanding the increasing amount of research, many discrepancies are still present in the determination of the antioxidant potency of the different plant extracts and essential oils, possibly due to different reasons. Plant extracts are traditionally obtained by steam distillation (essential oils) or by extraction with polar or nonpolar solvents (water, ethanol, methanol, acetone, hexane) and consequent solvent removal by evaporation[1]. The major components of the

Abbreviation used*: ABTS, 2,2'-azinobis(3-ethylbenzo-6-thiazoline-6-sulfonic acid); EBSS, Earl's balanced salt solution; FCS, fetal calf serum; FID, flame ionization detector; GAE, gallic acid equivalent; GC-MS, gas chromatography−mass spectrometry; HS, horse serum; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide; NADH, nicotinamide adenine dinucleotide (reduced form); SDE, simultaneous solvent extraction; TAA, total antioxidant activity; TC, α-tocopherol; TE, Trolox equivalent.*

essential oil and solvent extracts of the same plant are often very different, with consequent variation in their bioactive properties.

Furthermore, the different hydrophilic and lipophilic systems used for the evaluation of the antioxidant activity are not always predictive of the antioxidant potential in mammalian cell systems. Generally, these model systems appear rather suitable for a rapid comparative evaluation of the antioxidant power of plant sources. On the other hand, their evaluation in real food systems or, to an even greater extent, as potential agents to alleviate diseases by preventing oxidative deterioration, requires different scientific approaches.

In this study we evaluated the biological activity of aqueous extracts and essential oils from three cultivars of sweet basil (Ocimum basilicum L.) using primary cultures of rat cardiomyocytes as model system. The heart is an aerobic organ, and most of the energy required for the contraction and maintenance of ion gradients comes from oxidative phosphorylation. Oxidative stress caused by free radicals plays a crucial role in the pathophysiology associated with cardiovascular diseases; therefore, a great deal of attention has focused on the naturally occurring antioxidant phytochemicals as potential therapy.

The genus *Ocimum* L. includes approximately 150 species[2], with a great variation in plant morphology and biology, essential oil content, and overall chemical composition. Holy basil [Ocimum sanctum L. (syn. Ocimum tenuiflorum L.)] and sweet basil (*Ocimum basilicum* L.) are presently the two more considered species. *O. basilicum* is used as a culinary herb, but is also a well-known source of flavoring principles and scents. The leafy parts of basil have antimicrobial properties[3], are suitable for the treatment of pain and otitis [4], and have high antioxidant activity[5].

The essential oil derived from European basil, the major components of which are linalool and methylchavicol, according to the genotype[6], is considered to possess the finest aroma. Water-soluble extracts, which are known to contain phenolic compounds such as rosmarinic acid[7], are widely used as a preservative in the food industry due to the antioxidant activity of some of their constituents.

Many investigations on the antioxidant activity of basil oils and extracts have been carried out[8, 9], particularly regarding their antioxidant capacity in vitro. Tests in biological systems are, however, necessary to confirm their real effectiveness as protecting agents.

We investigated the effect of extract/oil supplementation in cultured cardiomyocytes in both normal conditions and after an oxidative stress, comparing it to the effect of 20 µM α-tocopherol (TC) supplementation. TC is considered to be the most important endogenous antioxidant in cardiac cells[10], and it is often used as positive control in studies regarding antioxidant protection in cultured cardiomyocytes.

The final aims of this study were to evaluate the biological activity of basil extracts, comparing it to the activity of a well-known antioxidant, and to verify if the different natures of extracts, as determined by the cultivar or extraction techniques, may affect their biological activities.

Materials and Methods

Materials

Horse serum (HS), fetal calf serum (FCS), Ham F10 culture medium, 2,2′-azinobis(3-ethylbenzo-6-thiazoline-6-sulfonic acid) (ABTS), Trolox, α-tocopherol, and other biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). α-Tocopherol (TC) was dissolved in ethanol at a concentration of 5 mM and kept at −20 °C

until use. All of the chemicals and solvents were of the highest analytical grade.

Basil seeds of three standard types:, Genovese, Lettuce leaf, and Purple, were obtained by a local seed company, currently using them as controls for breeding programs. Seeds were sown in alveolated plates with peat substrate at the end of March 2006. The plantlets were transplanted in the open field on a clay-loam soil, at the experimental farm of the University of Bologna, on May 19, in a two replication, randomized block design, as a part of a more extensive comparison of genotypes. Harvest took place on July 16, at preflowering stage. Mature leaves were removed from stems, separated into two subsamples, and immediately frozen. One subsample was lyophilized for extract preparation, and the other was used for essential oil extraction by distillation.

Methods

Aqueous Extracts and Essential Oils Preparation

For preparation of lyophilized water extracts, 25 g of lyophilized basil leaves, ground to a fine powder in a mill, were mixed by magnetic stirrer with 500 mL of boiling water and then filtered over Whatman no. 1 paper. The filtrates were frozen and lyophilized in a lyophilizer at 5 mmHg pressure and at −50 °C and then redissolved to solubilize the amount of lyophilized extract derived from 250 mg of fresh leaves in 1 mL of sterile bidistilled water. The obtained solutions were filtered on 0.20 µM filters (Whatman Europe, U.K.) and then used for cardiomyocytes supplementation.

The essential oil was obtained from 250 g of fresh frozen leaves by means of hydrodistillation and simultaneous solvent extraction (SDE) in a modified Likens−Nickerson apparatus[11], using diethyl ether as extraction solvent. This method is being routinely used in our research[12], because it is known to produce better recovery of all components with respect to ordinary hydrodistillation[13]. The solvent was removed under vacuum, at room temperature, and the amounts of essential oils were determined gravimetrically. The essential oils were then solubilized to dissolve the amount derived from 250 mg of fresh leaves in 1 mL of ethanol. The obtained solutions were filtered on 0.20 µM filters (Whatman Europe, U.K.) and then used for cardiomyocytes supplementation.

Aqueous Extracts and Essential Oils Analysis

Total phenol content of the aqueous extracts was determined according to ref[14], with slight modifications: 100 mg of lyophilized material was extracted with 0.99 mL of 80% (v/v) ethanol + 0.01 mL of 36% (v/v) HCl.

Essential oil composition was evaluated by gas chromatography (Perkin-Elmer Autosystem XL) combined with flame ionization detection (FID) and by GC-MS (Agilent 5973 Network Mass Selective Detector), using a BPX-5 (SGE Australia Pty-Ltd.) capillary column (95% polydimethylsiloxane + 5% phenyl, 30 m, 0.25 mm i.d., 0.25 µm d.f.). GC conditions were as follows: injection volume, 1 µL of 1/1000 (v/v) diethyl ether diluted essential oil; injector temperature, 240 °C; split ratio, 15/1; oven temperature program, 60 °C, increasing by 3 °C/min to 240 °C, held for 5 min. FID temperature was held at 280 °C. MS conditions were as follows: acquisition, full-scan mode; ionization energy, 70 eV; transfer line temperature, 240 °C; ion trap temperature, 210 °C. Essential oil components were identified by comparison of their retention times with those of pure standards, by means of Wiley and NIST/NBS mass spectra libraries, and by literature data[15].

Total Antioxidant Activity (TAA) of Extracts/Oils

TAA was measured using the method of Re *et al.*[16], based on the capacity of antioxidant molecules to reduce the radical cation of ABTS, determined by the decoloration of $ABTS^{\cdot+}$, and measured as the quenching of the absorbance at 734 nm. Values obtained for each sample were compared to the concentration−response curve of the standard Trolox solution and expressed as micromoles of Trolox equivalent (TE).

Cell Cultures

Primary cultures of cardiomyocytes were obtained from the ventricles of newborn Wistar rats according to the method of Yagev *et al.*[17]. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication 85-23, revised 1996). To obtain pure cultures avoiding the presence of fibroblasts, cells were preplated twice before the final seeding, and the absence of cells other than cardiomyocytes was verified microscopically. Cells were randomly divided in the control, grown in Ham F10 medium plus 10% fetal calf serum plus 10% horse serum, and supplemented cardiomyocytes, grown in the same medium but supplemented with different concentrations of extract solutions (1, 10, 20, or 40 µL mL^{-1} medium) or oil solutions (1 or 10 µL mL^{-1} medium), or with 20 µM TC. Media were changed every 48 h, the last medium change being 48 h before the experiments. At complete confluence in a monolayer, media were removed and substituted with Earl's Balanced Salt Solution (EBSS), pH 7.4 (116 mM NaCl, 5.4 mM KCl, 0.9 mM $NaH_2PO_4 \cdot 2H_2O$, 26 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4 \cdot 7H_2O$).

To cause oxidative stress, some cells received EBSS buffer added with 0.1 mM hydrogen peroxide. After 1 h, EBSS buffer was removed and collected for the determination of lactate dehydrogenase activity; cardiomyocytes were washed three times with 0.9% NaCl, and cell proliferative activity was assayed.

Lactate Dehydrogenase (LDH) Activity in Media

Cardiomyocyte plasma membrane integrity was assessed by measuring the release of LDH to the EBSS buffer. Enzyme activity was determined spectrophotometrically by measuring NADH levels at 340 nm[18].

Cell Proliferative Activity

Cell proliferative activity was measured using the 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay[19]. The test is based on the capacity of mitochondrial dehydrogenase in viable cells to convert MTT reagent to a soluble blue formazan dye. Briefly, after a washing with 0.9% NaCl, 1 mL of MTT reagent diluted in RPMI-1640 medium modified without phenol red (1 mg mL^{-1}) was added to each dish, and the cell cultures were incubated for 3 h at 37 °C. After removal of the medium, the cells were lysed with isopropanol for 15−20 min. Formazan production, which is proportional to cell vitality, was determined spectrophotometrically at 560 nm.

Statistical Analysis

The reported data are the means of at least three samples obtained from different cell cultures. Statistical analysis was carried out by means of one-way and two-way ANOVA. The differences between means were detected by means of Tukey HSD or Dunnett's test, in the case of comparisons with the untreated controls.

Results

Table 1 reports the extraction yield of aqueous extracts and essential oils, and the total phenolic contents of lyophilized aqueous extracts,

expressed as milligrams of gallic acid equivalent (GAE) per gram. The three basil types did not significantly differ for the amount of aqueous extract, whereas Purple basil contained almost twice the amount of phenolic compounds of Genovese and Lettuce leaf, in accordance with other results[20]. Genovese basil had significantly higher essential oil content than the two other types.

Table 1. Extraction Yield of Aqueous Extracts and Essential Oils and Total Phenolic Content of Lyophilized Aqueous Extracts[a]

type	aqueous extracts		essential oil content (mg g^{-1} of fresh leaves)
	sample amount (mg g^{-1} of fresh leaves)	total phenolic content (mg of GAE g^{-1} of lyophilized extract)	
Genovese	35.21±3.56	10.46±0.30 b	1.12±0.06 a
Lettuce leaf	34.31±4.99	10.04±0.38 b	0.81±0.09 b
Purple	36.37±2.99	20.57±0.34 a	0.69±0.03 b

[a]*Different letters indicate significant differences among types, according to Tukey's HSD, $p \leq 0.05$.*

Table 2 lists the chemical components of essential oils. The main constituent of the three types was linalool, significantly more abundant in Purple basil. Besides that, the essential oil composition of the three types was however different. Lettuce leaf oil was rich in methylchavicol, not present in the other two types, but had a substantially lower amount of eugenol. This component also differentiated Genovese, with the highest content, from Purple. Significant differences among types were also detected in the relative amounts of several less abundant components. These results are in agreement with those in the literature[21, 22].

In vitro total antioxidant activity (TAA) was calculated for the solutions obtained from aqueous extracts and essential oils and used for cell supplementation (Figure 1A). TAA, expressed as micromoles of TE per milliliter, was significantly lower in Lettuce leaf oil solution than in other oil solutions, whereas the corresponding extract had the highest TAA. These differences can be accounted for not only by the different phenolic content or qualitative composition of extracts/oils but also by the different extraction yields of the three cultivars. In fact, when the TAA of unit weight of pure oils or aqueous extracts was calculated (Figure 1B), Purple basil had the highest value, in clear relation to the highest content of phenolic compounds, as reported in Table 1.

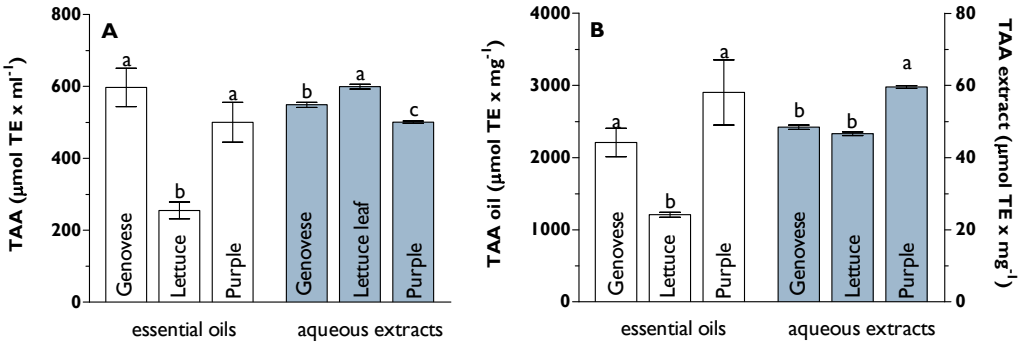
Regarding essential oils, Lettuce leaf had the lowest TAA, independent of the reference units, indicating that not only quantitative but also qualitative composition deeply influences in vitro TAA.

Table 2. Average Composition of the Three Examined Basil Essential Oils[a]

compound	content (% essential oil)		
	Genovese	Lettuce leaf	Purple
trans-2-hexenal	0.88 ± 0.03 a	0.64 ± 0.01 a	0.31 ± 0.10 b
cis-3-hexenol	0.22 ± 0.04 b	0.35 ± 0.01 a	0.23 ± 0.01 b
1-R-α-pinene	0.41 ± 0.08	0.35 ± 0.05	0.48 ± 0.00
camphene	0.06 ± 0.01	0.03 ± 0.04	0.09 ± 0.01
sabinene	0.40 ± 0.04	0.31 ± 0.03	0.35 ± 0.01
β-pinene	1.33 ± 0.14 b	1.14 ± 0.11 b	1.67 ± 0.01 a
β-myrcene	0.99 ± 0.13 a	0.58 ± 0.06 b	1.17 ± 0.01 a
α-terpinene	0.02 ± 0.03	0.02 ± 0.03	0.06 ± 0.01
limonene	0.35 ± 0.05 b	0.30 ± 0.05 b	0.64 ± 0.01 a
1,8-cyneol (eucalyptol)	8.10 ± 0.26	6.64 ± 0.60	7.90 ± 0.02
cis-ocymene	0.07 ± 0.00	0.04 ± 0.05	0.00 ± 0.00
1,3,6-ottatriene, 3,7-dimethyl (z)	1.60 ± 0.06 a	1.12 ± 0.07 b	0.06 ± 0.02 c
γ-terpinene	0.07 ± 0.01	0.10 ± 0.02	0.10 ± 0.01
cis-sabinene-hydrate	0.09 ± 0.01 a	0.00 ± 0.00 c	0.07 ± 0.01 b
linalool	38.27 ± 3.65 b	35.97 ± 0.57 b	47.46 ± 0.43 a
camphor	0.19 ± 0.08 b	0.32 ± 0.04 b	1.08 ± 0.01 a
β-fenchyl-alcohol	0.47 ± 0.01 a	0.19 ± 0.00 c	0.26 ± 0.01 b
1-terpinen-4-olo	0.18 ± 0.01	0.26 ± 0.04	0.24 ± 0.00
α-terpineol	1.09 ± 0.06 a	0.80 ± 0.04 b	1.18 ± 0.05 a
methyl-chavicol (estragol)	0.00 ± 0.00 b	33.07 ± 2.75 a	0.00 ± 0.00 b
fenchyl-acetate	0.00 ± 0.00 b	0.00 ± 0.00 b	0.19 ± 0.01 a
chavicol	0.02 ± 0.03 b	0.40 ± 0.06 a	0.00 ± 0.00 b
geraniol	0.08 ± 0.03 b	0.00 ± 0.00 b	0.95 ± 0.05 a
endo-borneyl-acetate	0.36 ± 0.07 a	0.19 ± 0.02 b	0.02 ± 0.02 b
eugenol	35.01 ± 3.99 a	9.36 ± 0.53 c	23.34 ± 0.82 b
β-elemene	0.22 ± 0.03 b	0.15 ± 0.01 b	0.40 ± 0.02 a
methyl-eugenol	0.09 ± 0.00 b	0.14 ± 0.01 a	0.18 ± 0.03 a
β-cariophyllene	0.00 ± 0.00 b	0.00 ± 0.00 b	0.72 ± 0.03 a
α-bergamotene	2.49 ± 0.07 a	1.27 ± 0.07 b	0.26 ± 0.03 c
α-guaiene	0.09 ± 0.01 b	0.00 ± 0.00 c	0.18 ± 0.01 a
δ-cadinene	0.09 ± 0.02 b	0.09 ± 0.01 b	0.16 ± 0.01 a
α-cariophyllene	0.14 ± 0.03 b	0.21 ± 0.01 ab	0.23 ± 0.00 a
β-farnesene	0.07 ± 0.00 b	0.00 ± 0.00 c	0.22 ± 0.00 a
epi-bicyclo-sesquiphellandrene	0.18 ± 0.01 b	0.16 ± 0.01 b	0.29 ± 0.01 a
germacrene D	0.85 ± 0.10	0.76 ± 0.01	0.80 ± 0.01
bicyclo-germacrene	0.41 ± 0.06	0.45 ± 0.00	0.39 ± 0.00
α-cubebene	0.29 ± 0.04 b	0.26 ± 0.02 b	0.43 ± 0.01 a
α-cadinol	2.24 ± 0.24 b	1.90 ± 0.06 b	3.12 ± 0.00 a

[a]*Only the identified components are reported. Within lines, different letters indicate among-varieties significant differences of compound relative content, according to Tukey's HSD, $p \leq 0.05$.*

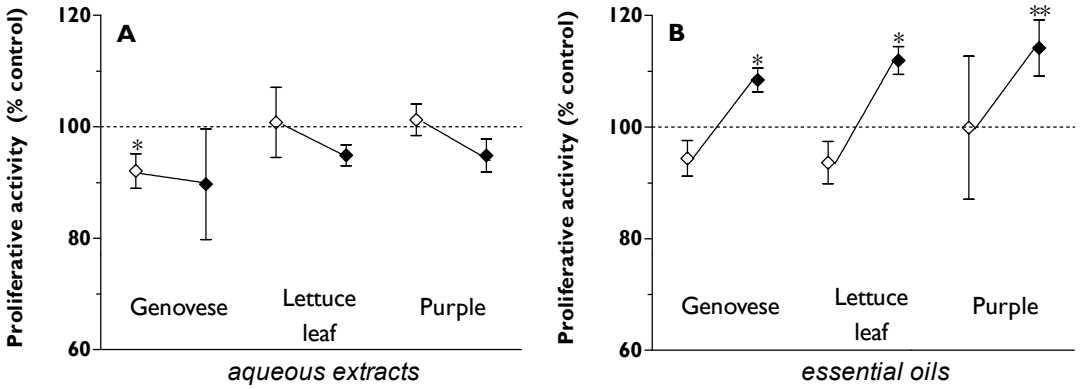
Figure 1. Total antioxidant activity (TAA) of basil essential oils and aqueous extracts. TAA is reported as micromoles of TE per milliliter (**A**) and micromoles of per milligram (**B**).



Data are means ± SD of three replicates. Different letters indicate significant differences ($p \leq 0.01$) between cultivars, within type of extract (essential oil or aqueous extract), according to Tukey's HSD.

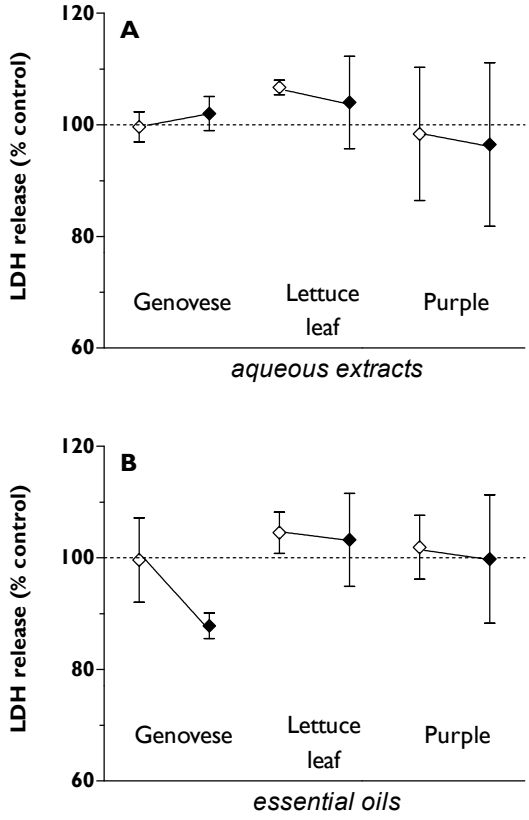
Cell proliferative activity in cardiomyocytes supplemented with basil oil and aqueous extract solutions at 1 and 10 µL mL^{-1} medium concentration is reported in Figure 2. Because basil oils were dissolved in ethanol, some control cells received the same amount of ethanol to exclude any interference due to the vehicle. Although ethanol addition did not modify either proliferative activity or LDH release (data not shown), supplemented cardiomyocytes were always compared to corresponding controls.

Figure 2. Proliferative activity in cardiomyocytes supplemented with basil aqueous extracts (**A**) and essential oils (**B**). Cardiomyocytes were supplemented with basil aqueous extracts and essential oils at two concentrations: 1 µL (◊) and 10 µL mL^{-1} of medium (◆).



*Data are expressed as percent of value in the corresponding control cells. *, **: significant differences with respect to the unsupplemented corresponding control (dashed line), according to Dunnett's test ($p \leq 0.05$ and ≤ 0.01, respectively).*

Figure 3. LDH release in cardiomyocytes supplemented with basil aqueous extracts (**A**) and essential oils (**B**). Cardiomyocytes were supplemented with basil aqueous extracts and essential oils at two concentrations: 1 µL (◊) and 10 µL mL^{-1} of medium (◆).



Data are expressed as percent of value in the corresponding control cells. No significant differences were detected with respect to the unsupplemented corresponding control (dashed line), according to Dunnett's test.

Cell proliferative activity was slightly decreased by Genovese aqueous extract supplementation, but the rather high data variability determined a significant deviation from the control only for the lower dose. Aqueous extracts from the other cultivars had no effect (Figure 2A). On the contrary, proliferative activity, although not affected by the lowest oil concentration, significantly increased in cardiomyocytes supplemented with the highest concentration, particularly of Purple basil oil (Figure 2B).

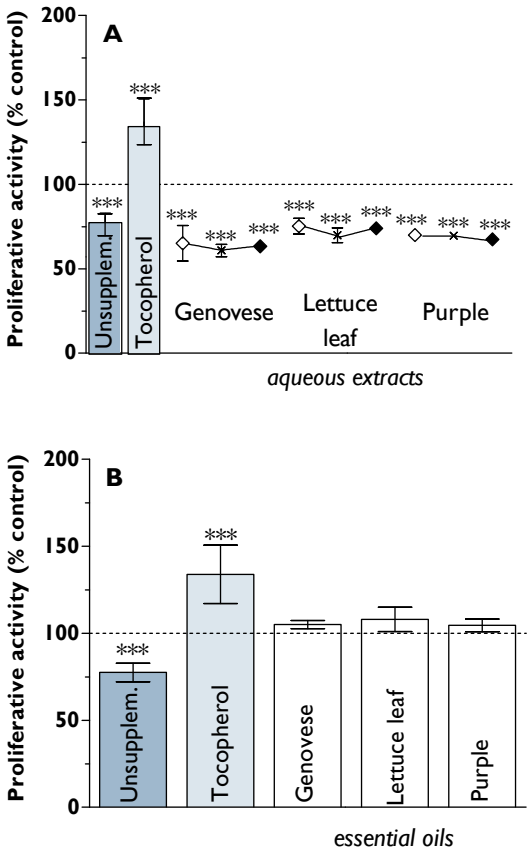
LDH release in cell medium is reported in Figure 3. Neither basil aqueous extracts nor basil oil supplementation caused modifications in LDH release, independent of the concentration.

To verify the biological protective effect of oils/extracts after an oxidative stress caused by the addition of hydrogen peroxide to the culture medium, cardiomyocytes were supplemented with solutions of basil oils and extracts at 10 µL mL^{-1} medium concentration. The results were compared to the protective effect obtained supplementing cells with a well-known antioxidant, 20 µM TC.

The addition of hydrogen peroxide to unsupplemented cells appeared to be cytotoxic, causing a significant decrease of cell proliferative activity, and a significant increase of LDH release with respect to nonstressed controls (Figures 4 and 5). Tocopherol supplementation exerted a complete protection against oxidative stress, as indicated by the increase in proliferative activity, which was even higher than in nonstressed controls, and by the prevention of LDH release.

The oxidative stress-induced reduction of proliferative activity was not counteracted by basil aqueous extract supplementation at 10 µL mL^{-1} medium (Figure 4A). Other experiments were then performed using higher concentrations (20 and 40 µL mL^{-1} medium); notwithstanding, basil aqueous extracts were unable to preserve proliferative activity in stressed cardiomyocytes. Conversely, basil essential oils at 10 µL mL^{-1} medium concentration showed a protective effect in stressed cells, proliferative activity being similar to that in nonstressed controls (Figure 4B).

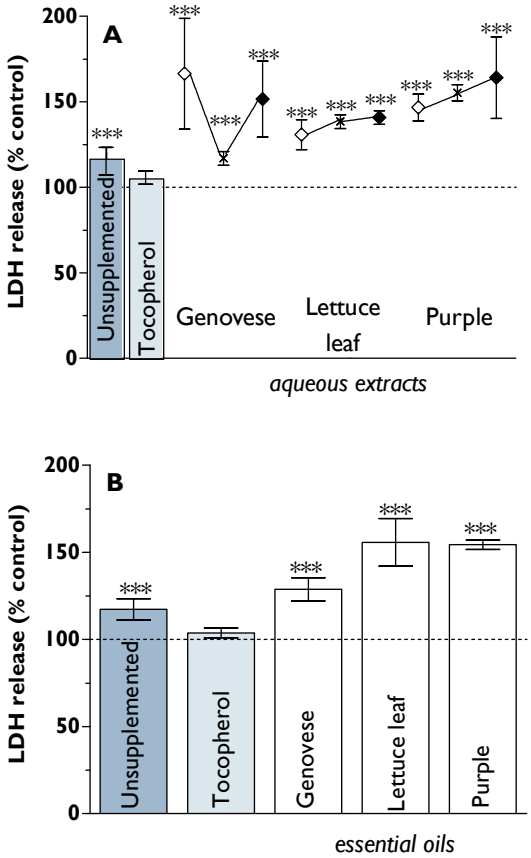
Figure 4. Proliferative activity in stressed cardiomyocytes supplemented with tocopherol, basil aqueous extracts (**A**) or basil oils (**B**). Cardiomyocytes were supplemented with basil aqueous extracts at three concentrations, 10 µL (◊), 20 µL (×), and 40 µL mL^{-1} of medium (◆) and with basil essential oils at 10 µL mL^{-1} of medium concentration.



*Data are expressed as percent of value in nonstressed unsupplemented cells. ***: significant difference with respect to nonstressed unsupplemented controls (dashed line), according to Dunnett's test ($p \leq 0.001$).*

In stressed cardiomyocytes the increase of LDH release was not prevented either by basil aqueous extracts or by oils (panels **A** and **B**, respectively, of **Figure 5**).

Figure 5. LDH release in stressed cardiomyocytes supplemented with tocopherol, basil aqueous extracts (**A**) or basil essential oils (**B**). Cardiomyocytes were supplemented with basil aqueous extracts at three concentrations, 10 µL (◊), 20 µL (×), and 40 µL mL^{-1} of medium (◆) and with basil essential oils at 10 µL mL^{-1} medium concentration.



*Data are expressed as percent of value in nonstressed unsupplemented cells. ***: significant difference with respect to the nonstressed unsupplemented controls (dashed line), according to Dunnett's test ($p \leq 0.001$).*

Discussion

It is well-known that most spices, especially those belonging to the Lamiaceae family, possess a wide range of biological and pharmacological activities. In addition to providing taste and flavor to foods, the antioxidant function of spices has been documented[23]. Dragland *et al.*[24] speculated that culinary herbs and spices contribute significantly to the total intake of antioxidants in a normal diet, even more effectively than a number of other food groups such as fruits, cereals, and vegetables.

In this study, the antioxidant activity of essential oils and water-soluble extracts from three cultivars of *O. basilicum* have been first evaluated in an in vitro test, and then their protective effect has been evaluated in cultured cardiomyocytes. To verify the effectiveness of supplemented oils/extracts in counteracting oxidative damage, cardiomyocytes were stressed by the addition of hydrogen peroxide.

As previously reported[25], we observed significant differences in the extraction yield of essential oil among the different cultivars, Genovese having the highest one. Because plants were grown in the same period and under the same conditions, these differences, as well as differences in essential oil composition, cannot be accounted for by environmental variations. According to ref[26], the linalool content characterized all cultivar oils, which differed in their content of eugenol (lower in Lettuce leaf) and methylchavicol (absent in Genovese and Purple).

The extraction yields of aqueous extracts were similar among cultivars, but total phenolic content of Purple was almost twice the content of the other cultivars.

To compare results, extracts and oils were solubilized to dissolve the amount of extract/oil derived from 250 mg of fresh leaves in 1 mL of water/ethanol, respectively, and then these solutions were used to supplement cardiomyocytes. Apart from Lettuce leaf oil, all solutions showed an in vitro TAA in the range of 500−600 µmol of TE mL^{-1}, Genovese appearing to be the most antioxidant among oils and Lettuce leaf among aqueous extracts. On the basis of the in vitro analysis, we could therefore suppose a similar biological activity for the different oils/extracts, apart Lettuce leaf oil.

When basil extracts/oils were supplemented to cardiomyocytes in basal condition, no cytotoxic effect was evidenced, in agreement with Zheljazkov *et al.*[27]; on the contrary, essential oil supplementation at the highest concentration significantly increased cell proliferative activity.

An antiproliferative activity for basil oils has been reported by Manosroi *et al.*[28] in a murine leukemia (P388) cell line, with an IC_{50} value of 0.0362 mg mL^{-1}. In our study, basil oil concentration ranged from 0.0017 (Purple) to 0.0028 (Genovese) mg mL^{-1} medium, being well below the toxic value. It is interesting to note that pro-proliferative activity was not present in cells supplemented with aqueous extracts and was not related to in vitro TAA, confirming that antioxidant activity in vitro does not predict biological effectiveness.

Differences among oils and extracts appeared to be even more evident in oxidative condition. Essential oil supplementation completely reversed the effect of hydrogen peroxide on proliferative activity, independent of the cultivar considered. On the contrary, extracts had no effect on this character, even at 4 times higher concentrations than essential oils.

With regard to LDH release, used to evaluate cell membrane integrity, neither oils nor extracts evidenced protective effect. Sharma *et al.*[29] reported a reduction of LDH release in the heart of infarcted rats after subcutaneous administration of a hydroalcoholic extract of *O. sanctum*, but it is

difficult to compare the two studies. Although a comparison with data obtained in other studies is out of the scope of the present work, which aimed at evidencing differences among oils and extracts derived from different cultivars of basil, the work of Sharma *et al.*[29] represents an important confirmation of the bioavailability of basil extracts and of the possibility of extract components to protect the heart.

Considering the proliferative activity, after oxidative stress basil oils appeared to be protective, whereas aqueous extracts had no effect. Major components of the essential oil and water-soluble extract are very different, and it is conceivable that they have different effects. It is interesting to note that, when the in vitro TAA of the solutions obtained from oils and extracts, which represented the solutions added to cardiomyocyte medium, was measured, extracts revealed similar or higher activity than oils, whereas they had no effect in cells. Our results underscore the need for biological measurement before protective activity is ascribed to plant material.

The highest protective activity of TC is not surprising because we used the most effective dose of the vitamin as positive control. The 20 μM dose has been reported to increase cellular TC content about 2−3-fold[30], to reduce membrane lipid alteration, and to actively protect cardiomyocytes from oxidative damage[31]. In a previous study the same dose of TC appeared to be more protective than extracts of green tea[31].

Although extraction techniques seem to be the main determinant of biological activity, with regard to oils, differences can be attributed even to cultivar. In fact, in basal condition, the Purple one was the most effective in enhancing proliferative activity. Basil cultivars differ in chemical composition, and it is conceivable that individual compounds may act differently, modulating different pathways, also in relation to specific experimental conditions.

The results obtained in this study clearly show that essential oils derived from *O. basilicum* have powerful protective activity against the antiproliferative activity due to oxidative stress, whereas water-soluble extracts are ineffective. Moreover, differences ascribable to cultivars have been detected. Notably, these results were not predictable simply by measuring in vitro TAA.

As far as we know, this is the first paper describing and comparing the biological effect of extracts/oils of different basil cultivars; our data may be useful for the consideration of basil both as a possible food ingredient or food supplement or in pharmaceutical applications. Further studies comparing the effects of basil extract to other antioxidants are needed, particularly considering that the quite recent knowledge that many botanical compounds can affect gene expression at the transcriptional level, thus regulating cell function[32], has widened the range of possible mechanisms of action of these compounds, which cannot be considered as mere antioxidants.

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Effect of green tea extract on cultured cardiomyocytes: from antioxidant activity to modulation and activation of transcription factors

Danesi F., Di Nunzio M., Boschetti E., Bordoni A. ***Green tea extract selectively activates peroxisome proliferator-activated receptor beta/delta in cultured cardiomyocytes****. Br J Nutr. 2008 5:1-4.*

Abstract

Hypoxia/reoxygenation is one of the causes of the increased expression of inducible NO synthase in cardiomyocytes. In a recent study we demonstrated that a single, high dose of green tea extract (GT) supplemented to the medium of cultured cardiomyocytes just before hypoxia/reoxygenation is able to prevent the increased expression of inducible NO synthase, therefore reducing NO overproduction. In the present study we investigated the mechanism by which GT reduces NO production. Since a molecular mechanism for polyphenol activity has been postulated, and PPAR activation is related to the transcription of the inducible NO synthase gene, we evaluated the activation of PPAR by GT. A moderate GT concentration, supplemented to the cardiomyocyte medium since the initial seeding, selectively activated the PPAR-β/δ isoform. Furthermore, we observed a reduction in NO production and an increase in total antioxidant activity, indicating that GT components may act on both reactive oxygen species, via an antioxidant mechanism, and NO overproduction. PPAR-β/δ activation could represent the key event in the reduction of NO production by GT. Although PPAR activation by GT was lower than activation by fenofibrate, it is very interesting to note that it was selective for the β/δ isoform, at least in neonatal cardiomyocytes.

Introduction

Tea, a preparation made from the dried leaves of *Camelia sinensis*, is a popular beverage all over the world; consequently, the significance of daily tea consumption and its possible healthy effects in humans are important issues. Most of tea's properties have been ascribed to its polyphenols, mainly catechins, which have been reported to possess antioxidant, anti-inflammatory and anti-cancer activity[1, 2].

Although promising experimental and clinical data demonstrated protective effects for the cardiovascular system, little is known about the mechanism of action of tea polyphenols at cellular and molecular levels. Evidence is accumulating that tea polyphenols can interfere with multiple

Abbreviation used*: CVDs: cardiovascular diseases; EGCG: epigallocatechin gallate; ELISA: enzyme-linked immuno-sorbent assay; FCS: fetal calf serum; GT: green tea; H/R: hypoxia/reoxygenation; HS: horse serum; iNOS: inducible nitric oxide synthase; LPS: lipopolysaccharide; NF-κB: nuclear factor-kappaB; NO: nitric oxide; PBS: phosphate buffered saline; PPARs: peroxisome proliferator activated receptors; RNS: reactive nitrogen species; ROS: reactive oxygen species; TAA: total antioxidant activity.*

pathways of signal transduction in cardiovascular relevant cells, and the induction of multiple effects may play crucial roles in the prevention and treatment of CVD[3]. The prevention of cardiovascular diseases and reduction of their mortality and morbidity remain some of the greatest public health challenges throughout the Western world.

It has been demonstrated that under pathophysiological conditions such as hypoxia with subsequent reperfusion, when the circumstances allow the formation of substantial amounts of reactive oxygen species and NO, these molecules react avidly and reactive nitrogen species are generated, causing damage to cellular components[4].

In a recent study[5], we demonstrated that the supplementation with a high concentration (about 150 µm-epigallocatechin-3-gallate (EGCG)) of a green tea extract (GT) to the medium of cultured cardiomyocytes just before the induction of hypoxia/reoxygenation is able to prevent the increase of inducible NO synthase expression. This could reduce NO overproduction; let us hypothesise that GT components act on both reactive oxygen species, via an antioxidant mechanism, and NO.

In the present study we investigated the mechanism by which GT reduces NO overproduction. Since a molecular mechanism for polyphenol activity has been postulated[6], and PPAR activation is related to inducible NO synthase expression[7], we evaluated the activation of PPAR by GT.

In order to verify the effect of chronic exposure to a moderate dose of GT, cardiomyocytes were grown since the initial seeding in a medium supplemented with a GT concentration corresponding to the EGCG plasma level related to the consumption of about five cups of green tea (about 30 µm-EGCG). After hypoxia/reoxygenation, NO production and total antioxidant activity were also measured.

Materials and methods

Materials

Horse serum, fetal calf serum, Ham F10, fenofibrate and other biochemicals were from Sigma Chemical Co. (St Louis, MO, USA). PPAR transcription factor assay kits were purchased from Cayman Chemicals (Ann Arbor, MI, USA). GT was from Indena (Milan, Italy) and was defined by the producer as having a polyphenol content of 75 ± 5 % (w/w), an EGCG content of 30 ± 5 % (w/w), a content of other catechins of 40 ± 10 % (w/w) and a caffeine content < 8 % (w/w) (by HPLC). GT was dissolved in warm bi-distilled water at the concentration of 1 mg/ml and kept at 4°C.

Methods

Primary heart cells were obtained from Wistar rats, aged 2–4 d, as reported[8], and grown until confluence in control medium (Ham F10 plus 10 % fetal calf serum plus 10 % horse serum) or in the same medium supplemented with GT (50 µg/ml). Media were changed every 2 d; the last change was 48 h before the experiment. Hypoxia was obtained by transferring cells to an air-tight, thermostated chamber (Bug Box; Ruskin Technology Ltd, Leeds, West Yorks, UK), as reported[8]. The hypoxia experiments lasted for 2 h, followed by 15 min reoxygenation. Then culture medium was removed, and NO production was evaluated in the medium using the Griess reaction after the construction of a standard curve by the use of known concentrations of sodium nitrite[9].

Cells were washed three times in PBS, and scraped off. Protein concentration was determined in each culture plate[10] to be sure that variables under study could not be affected by cell

number. Cytosolic and nuclear fractions were separated by differential centrifugation[11]. Total antioxidant activity was determined in the cytosol by the method of Re *et al.*[12]. PPAR activation was determined by ELISA utilising the PPAR transcription factor assay kits (Cayman Chemicals), following the manufacturer's instructions. Briefly, a specific double-stranded DNA sequence containing the peroxisome proliferator responsive element is immobilised onto the bottom of wells of ninety-six-well plates. Samples of nuclear extracts diluted to obtain 50 µg protein in each assay were added in each well, so that PPAR contained in the extract bind specifically to the peroxisome proliferator responsive element. Different PPAR isoforms were detected by the addition of specific primary antibodies directed against PPAR-α, -β/δ or -γ. A secondary antibody conjugated to horseradish peroxidase was added to provide a sensitive colorimetric readout at 450 nm.

Statistical analysis

Data are presented as mean values and standard deviations. Statistical analysis was by the Student's *t* test.

Results

As previously reported[13], hypoxia/reoxygenation dramatically increased NO production in control cardiomyocytes (Table 1). GT supplementation significantly reduced NO overproduction, according to the previously reported inhibition of inducible NO synthase overexpression[5].

After hypoxia/reoxygenation, as a consequence of NO and free radical overproduction, cytosolic antioxidant defences significantly decreased in control cardiomyocytes, while in GT-supplemented cardiomyocytes cytosolic total antioxidant activity appeared significantly higher than in basal, control cells (Table 1).

The nuclear extract derived from each sample was used to simultaneously evaluate the activation of all PPAR isoforms. Results obtained were expressed as the percentage of the value in control cells in basal conditions, considered as 100 %.

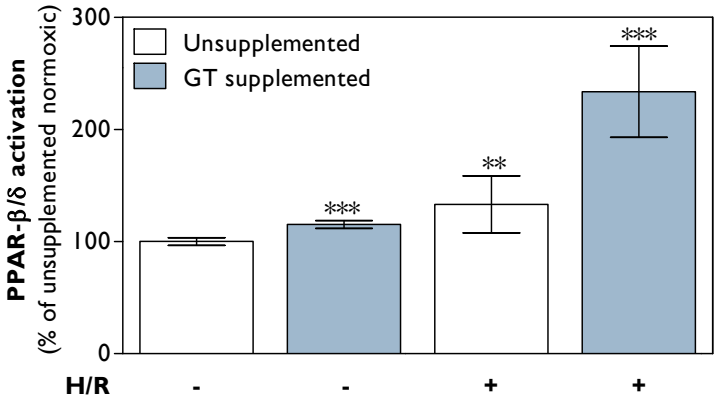
PPAR-α and PPAR-γ activation was not influenced either by hypoxia/reoxygenation or GT supplementation (data not shown). On the contrary, GT supplementation caused a significant PPAR-β/δ activation, particularly evident after hypoxia/reoxygenation (Figure 1). In order to better quantify the activation induced by GT on this PPAR isoform, in some experiments cells in basal conditions were stimulated for 4 h with 10 µm-fenofibrate, a pharmacological agent that ligands and activates PPAR. Either GT or fibrate significantly activated PPAR-β/δ (GT 115.12 (sd 3.49) %; fibrate 188.95 (sd 8.72) % of control; $P < 0.001$), although the effect of the pharmacological molecule was more evident.

Table 1. NO production and cytosolic total antioxidant activity (TAA) in control and green tea extract (GT)-supplemented cardiomyocytes, in basal conditions and after hypoxia/reoxygenation (H/R).

	NO (mmol/ml)	TAA (trolox equivalents/ml)
Control normoxic	38.45 ± 8.39	86.03 ± 4.54
GT normoxic	29.84 ± 5.21	85.60 ± 5.41
Control H/R	582.15 ± 50.55 ***	80.86 ± 4.88 *
GT H/R	148.92 ± 37.31**	98.64 ± 1.81***

*Mean value was significantly different from that of the control normoxic cardiomyocytes: *P < 0.05, **P < 0.01, ***P < 0.001.*

Figure 1. PPAR-β/δ activation in the nuclear extract of control and green tea extract-supplemented cardiomyocytes, in basal conditions (−) and after hypoxia/reoxygenation (+). PPAR-β/δ activation is expressed as a percentage of the value in control cells in basal conditions, assigned as 100 %.



Data are means of at least six samples obtained in different cell cultures, with standard deviations represented by vertical bars. Mean value was significantly different from that of the control cells in basal conditions: $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's t test).

Discussion

In the past decade, substantial progress has been made concerning our knowledge of bioactive components in plant foods and their links to health. Vegetable foods contain many hundreds of compounds which cannot be considered as nutrients, but appear to play a role in the maintenance of health. Bioactive components of edible vegetables include polyphenols, which display antioxidant properties. However, the bioavailability of potential antioxidants from plant foods is generally too low to have any substantial direct effect on reactive oxygen species, and it is postulated that the mechanism of action of polyphenols, even in very low concentrations, might entail their interaction with cell signalling and might influence gene expression, with the consequent modulation of several cell activities[14].

The present results indicate that a moderate dietary concentration of GT prevents NO overproduction and increases antioxidant defences, as indicated by cytosolic total antioxidant activity. The final result may be a counteraction of oxidative damage, which is the result of an imbalance between pro-oxidant and antioxidant molecules. GT appears to protect cells by reducing the former while increasing the latter.

Since it is conceivable that NO overproduction is prevented via the previously reported reduction of hypoxia/reoxygenation-induced inducible NO synthase overexpression, and Paukkeri *et al.*[7] recently demonstrated that PPAR agonists reduce lipopolysaccharide-induced inducible NO synthase expression and NO production in macrophages, we measured the effect of GT on the activation of the different PPAR isoforms.

GT supplementation had no effect on the α and γ isoforms, while it significantly activated the β/δ one, in both basal conditions and after hypoxia/reoxygenation. PPAR activation by GT

was lower than activation by fenofibrate, conceivable considering that fibrates are pharmacological agents.

Although both PPAR-α and -γ are expressed in neonatal rat ventricular myocytes[15], PPAR-β/δ is the predominant PPAR subtype in this cell type and plays a prominent role in the regulation of cardiac lipid metabolism[16]. It has been reported that PPAR-β/δ activation inhibits lipopolysaccharide-induced NF-κB activation[16]. Since activation of the transcription factor NF-κB facilitates the transcription of a number of genes such as inducible NO synthase[17], activation of PPAR-β/δ could represent the key event in the reduction of NO production by GT.

At present, it is difficult to distinguish whether PPAR activation by GT is due to direct catechin binding to the transcription factor or it is caused by an increased availability of PPAR-β/δ. EGCG has been reported to inhibit proteasomal activity[18], which, in turn, regulates the availability of transcription factors such as PPAR[19]. Notwithstanding, it is very interesting to note that GT selectively activated the β/δ isoform, at least in neonatal cardiomyocytes.

Further studies are needed to better understand the molecular effects of GT in cardiomyocytes, but data reported in the present study appear promising, not only because it is demonstrated that an aberrant NO production has detrimental consequences and is involved in the pathophysiology of myocardial infarction[20], but also considering the possible effect of GT as a PPAR-β/δ selective activator. Very recently, Sheng *et al.*[21] demonstrated that GW0742, a synthetic selective agonist of this PPAR isoform, exerts a beneficial effect on angiotensin II-induced cardiac hypertrophy. In our knowledge there are no data in the literature regarding PPAR activation by green tea, apart from the study by Lee[22] on mouse cloned PPAR-α. Other studies are needed to verify if GT selective activation of PPAR-β/δ is a feature limited to neonatal rat cardiomyocytes or more generally to cardiac cells. Maybe in the near future modern medicine will be able to better explain why tea was considered the 'long-life beverage' by ancient medicine.

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M.D.N. and E.B. performed the analysis together with F. D., who also did the data analysis and contributed to the drafting of the paper; A.B. designed and supervised the study, and wrote the paper.

All authors state that there is no conflict of interest associated with the present study.

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Nutritional modulation of interleukin-23 receptor and regulation of derived pro-inflammatory cytokines as potential regulator of bowel inflammation

Danesi F., Philpott M., Huebner C., Bordoni A., Ferguson L.R. ***A screen to test the role of IL-23 receptor in inflammatory bowel diseases****. Journal of Nutrigenetics and Nutrigenomics. 2008; 1(6): 305.*

Abstract

Several studies have established that nutrient/gene interactions modulate inflammation, producing either positive or negative effects depending on the net changes in gene expression. To date, few studies have been devoted to elucidating mechanisms underlying this modulation of the inflammatory response in the gut.

The purpose of this study was to develop an assay to investigate the effects of specific micronutrients on inflammatory bowel biomarkers. Kit 225 cells, a human T cell line, were used as a model system. Kit 225 cells have similarities to Th17 cells, a subset of T cells producing interleukin-17 (IL-17). IL-17 is an important mediator of the T-cell response in inflammatory bowel disease (IBD). First, the expression of the interleukin-23 (IL-23) receptor complex, involved in regulation of IL-17 release, was established. Then IL-17 mRNA and protein expression were evaluated in our model system. The involvement of other pro-inflammatory mediators, such as tumor necrosis factor- α (TNF-α), was also verified. IL-23 (1 ng/ml medium for 24 hours) increased the expression of IL-17 mRNA in the cells more than three-fold, relative to the untreated controls. Similarly, TNF-α expression was significantly increased in the IL-23-treated cells.

The influence of different food bioactives on IL-17 and TNF-α mRNA expression and on IL-17 production was evaluated. The selected tested bioactives were: caffeic acid phenethyl ester (CAPE), epigallocatechin gallate (EGCG), docosahexaenoic acid (DHA), and linoleic acid (LA). These experiments elucidate the effect of specific micronutrients on IL-23 receptor activity.

Abbreviation used*: 5-ASA: 5-aminosalicylic acid; CAPE: caffeic acid phenethyl ester; DHA: docosahexaenoic acid; DMEM: Dulbecco's modified Eagle's medium; EGCG: epigallocatechin gallate; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GSH: glutathione (GSH); IBD: inflammatory bowel disease; IFN-γ: interferon-γ; IL: interleukin; IL-12 Rβ1: interleukin-12 receptor- β1; IL-12: interleukin-12; IL-17: interleukin-17; IL-2: interleukin-2 (IL-2); IL-23 R: interleukin-23 specific receptor; IL-23: interleukin-23; LA: linoleic acid; LPS: lipopolysaccharide (LPS); MAPKs: mitogen-activated protein kinases; Na Sal: sodium salicylate; NF-κB: nuclear factor κB; PCR: polymerase chain reaction; PKC: protein kinase C; PMA: phorbol myristate acetate; PPAR: proliferator-activated receptor; TNF-α: tumor necrosis factor- α.*

Our findings suggest that different food bioactives can regulate IL-23 receptor activity, that plays a pivotal role in the modulation of proinflammatory cytokines involved in IBD; therefore IL-23 receptor is representing a key target for a nutritional intervention in inflammation disease.

Introduction

Inflammatory bowel disease (IBD) is a complex multi-factorial disease that involve interactions between host genetic and environmental factors. Although the etiology of IBD is unknown, results from clinical and experimental studies indicate a breakdown in intestinal homeostasis with the development of aberrant inflammatory responses to intestinal bacteria[1].

The possibility of dietary intervention strategies in IBD has long been hypothesised. The recognition of the association between IBD and intestinal microflora[2] has led to clinical studies investigating the therapeutic potential of altering the luminal bacteria using probiotics or prebiotics. Despite promising results with animal models, the efficacy of probiotic use in humans with IBD is unclear[3]. Synbiotics, which is the combination of probiotics and prebiotics, is a novel idea to maximize the favorable intestinal environment, but larger, controlled trials including species-specific studies must be performed[3].

Another interesting possibility could be the modulation of inflammatory cytokines by food bioactives.

Among the inflammatory cytokines implicated in IBD pathogenesis, recent attention has focused on the interleukin-12 (IL-12)-related cytokine IL-23 as a key driver of intestinal inflammation[4-8]. IL-23 is a pivotal player in intestinal homeostasis via its ability to orchestrate both T-cell-dependent and innate pathways of intestinal inflammation and to suppress regulatory T-cell responses in the intestine.

IL-23 receptor is a heterodimer sharing one subunit, IL-12Rβ1 (binds IL-12p40), with the IL-12 receptor, awhile the other subunit, IL-23R, is specific. IL-23-receptor is expressed in T cells, natural killer cells, dendritic cells, and macrophages[9].

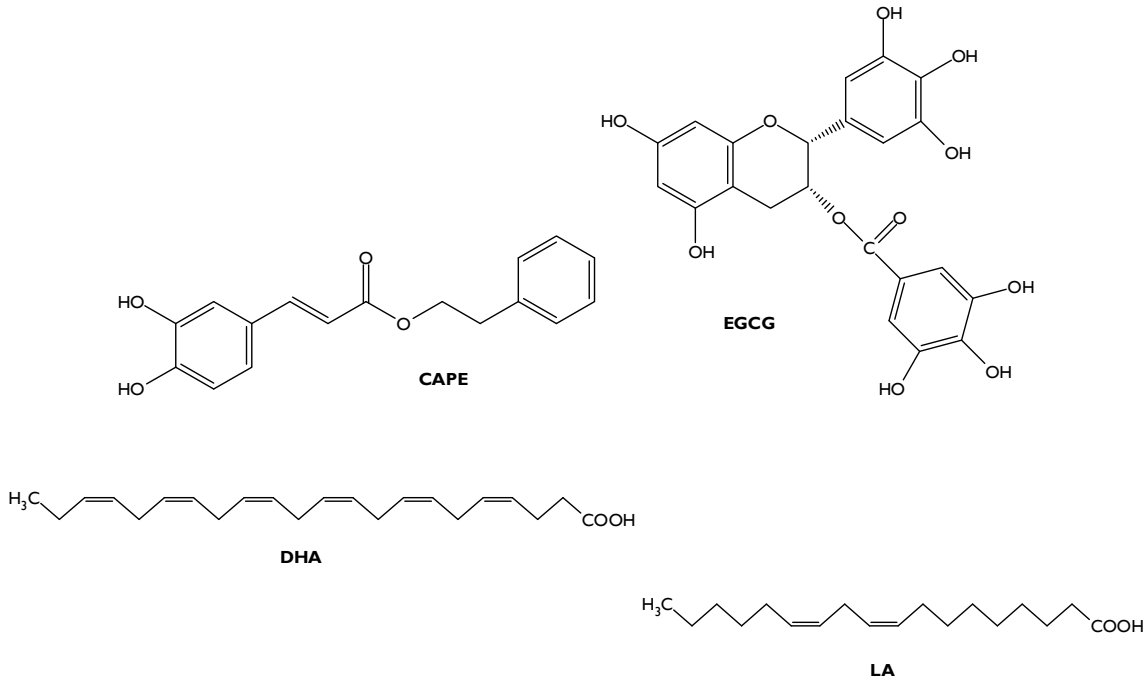
IL-23 induces interferon-γ (IFN-γ) production from activated T cells[10], and recent work has focused on its ability to promote a novel subset of IL-17-producing $CD4^+$ helper T cells termed Th17 cells[11]. Th17 cells are a heterogeneous population producing a number of cytokines in addition to IL-17 including IL-6, IL-17F, IL-21, IL-22, and TNF-α[12].

The ability of IL-23 to differentially induce intestinal inflammation suggests the presence of tissue-specific IL-23-dependent effector pathways. In innate colitis, IL-23 was found to control the production of a number of inflammatory cytokines such as IL-1β, IL-6, TNF-α and IFN-γ[7]. In addition, in both anti-CD40 and *H. hepaticus*-induced colitis, there was a highly localized induction of the Th17 cytokines IL-17, IL-17F, and IL-22[5] in the intestine.

In this work, we have evaluated the influence of different food bioactives on IL-17 and TNF-α mRNA expression and on IL-17 production. Kit 225 cells, a human T cell line possessing similarities to Th17 cells, were used as a model system.

Three of the tested bioactives (**figure 1**), namely caffeic acid phenethyl ester (CAPE), epigallocatechin gallate (EGCG), and docosahexaenoic acid (DHA), were chosen since their ability to reduce inflammation modulating cytokine pattern is reported in literature[13-15]. We also tested linoleic acid (LA) (**figure 1**) since this essential fatty acid is precursor of both γ-linolenic and arachidonic acid, which are considered anti- and pro-inflammatory, respectively[16, 17].

Figure 1. The chemical structure of caffeic acid phenethyl ester (CAPE), epigallocatechin gallate (EGCG), docosahexaenoic acid (DHA), and linoleic acid (LA).



Materials

T-cell lymphocytic leukemia cell line (Kit 225) was the kind gift of Prof. Frank Ruscetti (National Cancer Institute, National Institute of Health, USA), while the human colon epithelial cancer cell line (Caco-2) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Interleukin-2 (IL-2) and interleukin-23 (IL-23) were purchased from R&D Systems (USA). All other chemicals and solvents were purchased from Sigma Chemical (USA).

Cell Culture

Kit 225 and Caco-2 cell lines were cultured separately and maintained in a 37°C humidified incubator containing 5% CO_2. Kit 225 cells were maintained in RPMI 1640 culture medium containing 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and supplemented with 20 ng/mL recombinant human interleukin-2 (IL-2). Cells were passaged weekly. Caco-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) in the presence of 10% fetal bovine serum supplemented with 1 mM sodium pyruvate and 1 mM non essential amino acids and 50 µg/mL gentamycin.

Gene expression studies

In preliminary experiments, the expression of the functional IL-23 receptor complex was evaluated with quantitative real time PCR in both Kit 225 and Caco-2 cells. At 90% confluence, Kit 225 cells were transferred into 25 cm^2 flasks and stimulated with 0, 1 or 10 ng/mL interleukin-23 (IL-23) for 24 hours. IL-23 was diluted in PBS to a stock solution of 10 µg/mL, aliquoted and stored at -20°C. Stock solution was diluted with medium to the desired concentrations immediately before use. Comparative experiments were performed simultaneously in Caco-2 cells, not stimulated with IL-23.

For testing the effects of dietary supplements, Kit-225 cells (90% confluence) were supplemented with 10 µg/mL medium caffeic acid phenethyl ester (CAPE), epigallocatechin gallate (EGCG), docosahexaenoic acid (DHA), or linoleic acid (LA) (as free acids). Phorbol myristate acetate (PMA) and sodium salicylate (Na Sal) (10 µg/mL medium) were used as pro-and anti-inflammatory controls, respectively. CAPE, EGCG, DHA, LA and PMA were resuspended in dimethyl sulfoxide (DMSO) to a concentration of 1 mg/mL, and an equal amount of Milli-Q water added. Each solution was filtered through 0.2 µm nylon syringe filters (Corning, USA), aliquoted and stored at -20°C. Sodium salicylate was dissolved in Mill-Q water to give a 100 mM solution, which was filter-sterilized before use. 24 hours after supplementation, cells were stimulated with 1 ng/mL IL-23 for further 24 hours. Cells were counted with a hemocytometer to ensure comparable cell numbers for each experiment.

Total RNA extraction and real time-PCR

Total RNA was isolated from Kit 225 and Caco-2 cultured cells with the Mini Rneasy kit (Qiagen, USA), treated with DNase (Qiagen), and quantitated by calculation of the optical density at 260 nm with an ND-1000 Spectrophotometer (NanoDrop, USA). RNA was transcribed to cDNA with random hexamers and the SuperScript III First-Strand Synthesis SuperMix (Invitrogen, USA), following manufacturer's instructions. The resulting cDNA was stored at -80°C.

Absolute quantification of gene expression of selected cytokines (IL-17, TNF-α and IFN-γ) was performed using the ABI PRISM 7900HT Sequence Detection System (Perkin Elmer, USA). Specific primer pairs were designed by using Oligoperfect Designer (http://www.invitrogen.com/) (**Table 1**). Real time PCR amplification was carried out in 9.0 µl reaction volume in a 384 well optical PCR plate (Perkin Elmer, USA), using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, USA). 1 µl of the template cDNA was included in each reaction volume except for the negative controls. Aliquots were then amplified by an initial period of 2 min at 50°C and 5 min at 95°C followed by 40 concurrent cycles involving denaturation (at 95°C for 15 s), annealing (at 58°C for 30 s) and extension (at 72°C for 30 s), further followed by a melting curve analysis (95°C for 15 s, 60°C for 15 s, and 95°C for 15 s) with continuous monitoring of fluorescence. Reactions were typically run in duplicate. Gene expression was normalized to the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). Real-time data was analyzed with SDS software, version 2.3 (Applied Biosystems, USA). The data was presented as a mean fold change of relative expression as compared to that seen in control cells (normalized to 1).

Table 1. Primer sequences used in real-time PCR assay.

Gene	Primer sequence
IL-23 R	Forward 5'-ACACATGGAATTCTGGGCTA-3' Reverse 5'-TAATTCCAGTGAGCAGGTCC-3'
IL-12 Rb1	Forward 5'-GCGGACCTCAGGATGATG-3' Reverse 5'-TCCACTTGCTCCAGGAACTT-3'
IL-17	Forward 5'-TCATCCATCCCCAGTTGATT-3' Reverse 5'-TTCGTGGGATTGTGATTCCT-3'
TNF-α	Forward 5'-TCAGCCTCTTCTCCTTCCTG -3' Reverse 5'-GCCAGAGGGCTGATTAGAGA-3'
GAPDH	Forward 5'-CAAGGAGTAAGACCCCTGGA-3' Reverse 5'-GGGTCTACATGGCAACTGTG-3'

IL-23 R: interleukin-23 specific receptor, IL-12 Rb1: interleukin-12 receptor beta 1, IL-17: interleukin-17, TNF-α: tumor necrosis factor-alpha, GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

Determination of IL-17 production

Measurement of interleukin 17 (IL-17) production in cell culture media was performed using Quantikine ELISA kits (R&D System, USA), following the manufacturer's protocol. Briefly, a specific monoclonal antibody was pre-coated onto the bottom of 96 well microplate. Standards (recombinant human IL-17 diluted in Milli-Q water, ranging from 30 to 2000 pg/mL) and samples were pipetted into the wells, so that the IL-17 contained in the supernatant could bind specifically to the immobilized antibody. A substrate solution was added to provide a sensitive colorimetric readout at 405 nm. By comparing the optical density of the samples to the standard curve, the IL-17 concentration in the culture supernatant was determined, and expressed as pg/mL.

Statistical analysis

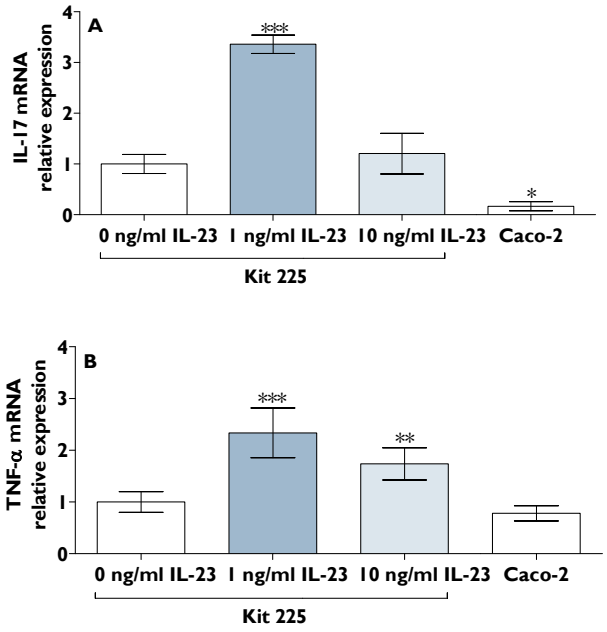
Statistical significance was evaluated by an unpaired Student's t-Test using the GraphPad Prism software, comparing supplemented cells with their respective control.

Results

The preliminary evaluation of the expression of the functional IL-23 receptor complex by quantitative real time PCR revealed a significantly higher expression of both subunits, IL-23 specific receptor and IL-12 receptor beta 1, in Kit 225 cells with respect to Caco-2 *(data not shown)*.

IL-17 gene mRNA expression was evaluated in Kit 225 cells in basal condition and upon stimulation with two different concentration of IL-23, and in Caco-2 cells for comparison. As reported in **figure 2**, IL-17 gene was expressed in Kit 225 cells, but not in Caco-2 ones, in basal condition,

Figure 2. Effect of IL-23 stimulation on IL-17 (A) and TNF-α (B) gene expression in Kit 225 cells.



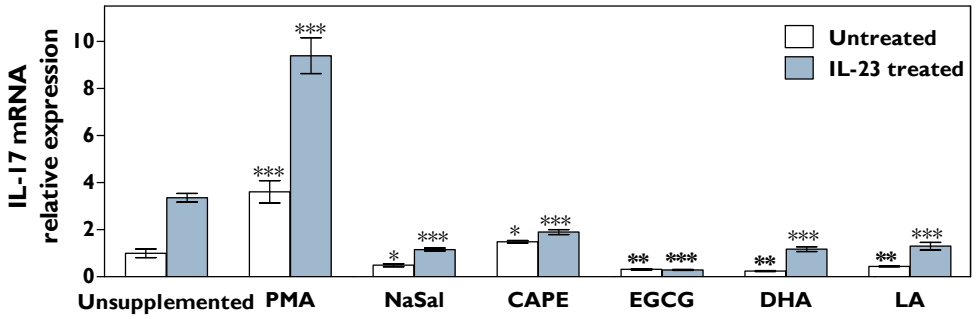
Data are presented as the mean fold change (± SD) of relative expression compared to unstimulated (normalized to 1). For details see Methods. Caco-2 cells were used as comparison. Significant differences were evaluated by t-test in comparison with unstimulated Kit 225 cells (p<0.05, ** p<0.01, *** p<0.001).*

and appeared upregulated by the low concentration of IL-23. On the contrary, Il-23 highest concentration did not modify IL-17 mRNA expression.

The basal expression of TNF-α encoding gene was similar in Kit 225 and Caco-2 cells. Again, IL-23 stimulation up-regulated gene expression, particularly when the lower dose was used.

The effect of the different supplementations on the mRNA expression of IL-17 gene in both basal condition and after IL-23 stimulation is reported in figure 3. In both conditions, the pro-inflammatory PMA caused the over-expression of IL-17, which appeared down regulated in cells supplemented with the anti-inflammatory Na-Sal. A similar or even higher anti-inflammatory effect was achieved supplementing cells with EGCG, DHA and LA. CAPE supplementation slightly upregulated IL-17 gene in the absence of IL-23 stimulation, while after stimulation it caused a downregulation in comparison to the respective unsupplemented control.

Figure 3. IL-17 mRNA expression in unsupplemented and supplemented Kit 225 cells in basal condition and after IL-23 stimulation.

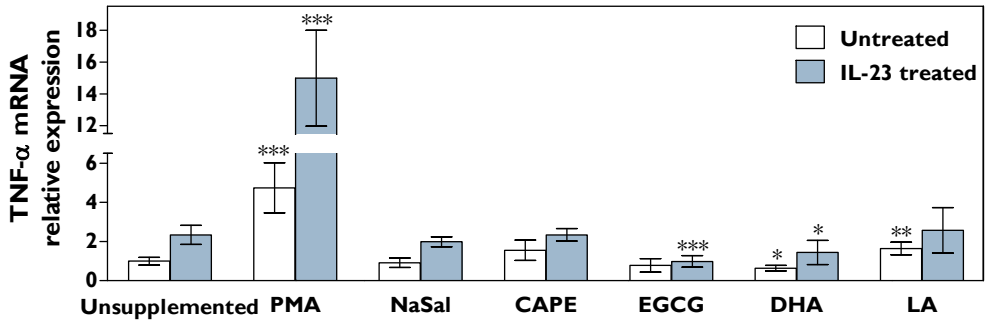


Data are presented as a mean fold change (± SD) of relative expression compared to unsupplemented cells in basal condition (normalized to 1). For details see Methods. In both conditions (unstimulated - white bar, and stimulated - blue bar) significant differences were evaluated by t-test in comparison with the corresponding unsupplemented Kit 225 cells (p<0.05, ** p<0.01, *** p<0.001).*

As shown in figure 4, PMA caused the over-expression of TNF-α gene, too, while Na-Sal was ineffective, as well as CAPE. In both unstimulated and stimulated conditions DHA significantly downregulated gene expression, while the effect of EGCG and LA appeared to be dependent on the presence of IL-23, the first one being anti-inflammatory only in stimulated condition, the second one pro-inflammatory only in basal one.

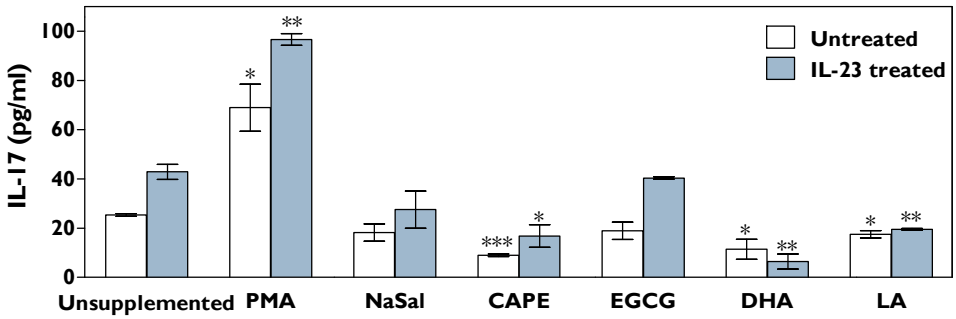
The molecular effect of PMA , DHA and LA were completely in agreement with data regarding IL-17 protein expression (figure 5). Conversely, no modification in IL-17 protein concentration was detected in Na-Sal and EGCG supplemented cells compared with the corresponding unsupplemented ones. Regarding CAPE supplementation, in stimulated condition the lower protein concentration reflected the downregulation of the encoding gene, while in basal condition the increased gene expression was accomplished to a reduced protein synthesis.

Figure 4. TNF-α mRNA expression in unsupplemented and supplemented Kit 225 cells in basal condition and after IL-23 stimulation.



Data are presented as a mean fold change (± SD) of relative expression compared to unsupplemented cells in basal condition (normalized to 1). For details see Methods. In both conditions (unstimulated - white bar, and stimulated - blue bar) significant differences were evaluated by t-test in comparison with the corresponding unsupplemented Kit 225 cells (p<0.05, ** p<0.01, *** p<0.001).*

Figure 5. IL-17 production in unsupplemented and supplemented Kit 225 cells in basal condition and after IL-23 stimulation.



Data are presented as a mean (± SD) of IL-17 protein concentration in cell culture supernatant (pg/ml). For details see Methods section 2.4. In both conditions (unstimulated - white bar, and stimulated - blue bar) significant differences were evaluated by t-test in comparison with the corresponding unsupplemented Kit 225 cells (p<0.05, ** p<0.01, *** p<0.001).*

Discussion

In humans, interleukin-23 (IL-23) receptor is expressed in activated/memory T cells, NK cells, macrophages, DC and monocytes[9, 10]. IL-23 receptor has a key role in the regulation of a newly defined effector T-cell subset, Th17 cells, a pathogenic subset characterized by the production of interleukin-17 (IL-17)[12, 18, 19]. Recently, IL-23 and Th17 cells have been implicated in several aspects of autoimmune disease[20]; particularly, IL-23 has emerged as a key

player in chronic intestinal inflammation as shown in several models of inflammatory bowel disease (IBD)[5, 6, 8, 21-25].

In this paper we evaluated the possibility of modulating IL-23 receptor by supplementing cells with different bioactive food components.

We used Kit 225 cells as novel model system. Kit 225 cells, an interleukin 2 (IL-2)-dependent human T cell line derived from chronic lymphocytic leukemia[26], have similarities to Th17 cells, and Parham *et al.*[9] demonstrated that they have a proliferative response to IL-23. In preliminary studies we demonstrated that Kit 225 cells express both subunits (IL-12 beta 1 and IL-23 R) of IL-23 receptor. In this study we evidenced that IL-23 treatment lead to up-regulation of the expression of some important mediators of the T-cell response in IBD, i.e. IL-17 and TNF-α.

Cytokine gene expression was evaluated 24 h after IL-23 stimulation, a lag time widely accepted[27]. In unsupplemented Kit 225 cells IL-17 mRNA levels increased more than three-fold upon 1 ng/ml IL-23 exposure, in agreement with other authors[18, 28], while 10 ng/ml IL-23 were ineffective. This could be explained by an early change in gene expression consequent to the higher dose, followed by the return to baseline after 24 h. TNF-α mRNA expression was similarly up-regulated by both IL-23 concentrations. Even in this case, it is conceivable that stimulation with the high dose of IL-23 caused a very fast upregulation of TNF-α gene, which expression was coming back to basal value after 24 h. Although further studies are needed to clarify this point, our data clearly indicate that kit 225 cells possess a functional IL-23 receptor, and are able to produce IL-17 and TNF-α following IL-23 exposure. IL-17 and TNF-α are key cytokines playing redundant and pleiotropic effects contributing to the inflammatory response. It has been demonstrated that IL-17 and TNF-α over-expression induced by phorbol myristate acetate (PMA) lead to activation of mitogen-activated protein kinases (MAPKs) via the protein kinase C (PKC) pathway[29]. Over-expression of IL-17 and TNF-α is probably depending on activation of nuclear factor κB (NF-κB)[30, 31], which is up-regulated in macrophages and epithelial cells of IBD patients[32]. Sodium salicylate (Na Sal), a known anti-inflammatory, acts through suppression of NF-κB activity[33].

In our study PMA supplementation increased significantly IL-17 mRNA expression, IL-17 protein production and TNF-α mRNA expression. Moreover, it acts synergistically with IL-23 in increasing these parameters. On the opposite, sodium salicylate was significantly effective only in preventing IL-17 gene over-expression, but not IL-17 protein overproduction or TNF-α gene up-regulation.

All bioactives tested in this study can be find in foods: caffeic acid phenethyl ester (CAPE) is a phenolic component of the propolis obtained from honeybee hives, epigallocatechin gallate (EGCG) is the main polyphenol found in green tea, docosahexaenoic acid (DHA) is the fatty acid which characterized fish lipids, and linoleic acid (LA) is widely present in diet, mainly in vegetable oils. Some of them (CAPE, EGCG, and DHA) have been shown to be very potent anti-inflammatory agents both *in vitro* and *in vivo*[34-37].

Regarding IL-17 gene mRNA expression our data confirmed EGCG and DHA anti-inflammatory activity in both treated and IL-23 untreated cells, while CAPE downregulated it in treated cells only. The inhibition of IL-17 mRNA expression by these bioactives is probably due to suppression of NF-κB activation, as demonstrated by other authors in different model systems[38-40]. IL-17 downregulation following LA supplementation was unexpected, since LA is considered a proinflammatory agent acting via activation of NF-κB and MAPK cascade[41, 42]. Since LA is substrate for the synthesis of both γ-linolenic and

arachidonic acid, which are precursors of anti- and pro-inflammatory eicosanoids respectively, our results could depend on the prevalence of γ-linolenic acid production from LA in our experimental condition.

Comparing IL-17 mRNA expression analysis with protein expression profile, we evidenced a good correlation between these two parameters in CAPE, DHA and LA supplemented cells, as also verified by other authors[43-46]. This good correlation between IL-17 mRNA level, quantified by real-time PCR, and protein level, quantified by enzyme-linked immunosorbent assay, is probably due to the short half-life of cytokines. In fact, cytokine secretion depends on new protein synthesis, and the elaboration of cytokine response to inflammatory stimuli is ultimately regulated by the transcription rates of cytokine genes[47]. Regarding EGCG, downregulation of IL-17 gene mRNA expression was not accomplished with a decrease in protein synthesis. Further studies are needed to explain this result.

Regarding TNF-α gene expression, although various investigators have demonstrated that CAPE has anti-inflammatory properties both *in vitro* and *in vivo*[32, 48-51], in our study it was ineffective either in absence or presence of IL-23 treatment. Similar observations have been already reported in alveolar epithelial cells, where CAPE did not totally abrogate lipopolysaccharide (LPS)-mediated TNF-α biosynthesis[40, 52]. CAPE has been demonstrated to be a specific inhibitor of the NF-κB, this accounting for its anti-inflammatory actions[53]. It must be considered that TNF-α gene contains not only NF-κB binding sites, but also many other potential regulatory elements. In addition, MAPK regulation has been recently involved in the regulation of proinflammatory cytokine biosynthesis and transcription[54]. Since cross-talk between MAPK and NF-κB signaling pathways is probable, all of these pathways may play a role in regulating TNF-α mRNA expression after inflammatory stimulus. The inhibition of NF-κB alone may therefore be not sufficient for a significant reduction of the TNF-α over-expression due to IL-23. Further studies are necessary to evaluate CAPE effect on NF-κB inhibition and TNF-α protein production in IL-23-treated cells.

EGCG has been demonstrated to inhibit LPS-induced TNF-α production in mouse macrophages[55, 56]. Our results confirmed the downregulation of TNF-α mRNA expression also in IL-23-stimulated Kit 225 cells. It is conceivable that also this down-regulation is due to the inhibition of the activation of NF-κB [30, 56-59].

Several studies suggest that n−3 PUFAs are potent anti-inflammatory, that act by down-regulation of NF-κB activity, both in animal and other cell models. In this study we evaluated the individual effect of DHA, without the contribution of EPA. Notwithstanding EPA anti-inflammatory activity related to its eicosanoid derivatives, DHA has been demonstrated to reduce TNF-α mRNA expression greater than EPA in a different system model[60]. Actually, DHA suppressed significantly the TNF-α mRNA expression also in Kit 225 cells. This effect could be ascribed to an alteration of the cellular redox status, which could in turn affect NF-κB activity. Komatsu *et al.*[61] demonstrated that DHA reduces oxidative stress and increases levels of the antioxidant glutathione (GSH), so suppressing nitric oxide production and inducible nitric oxide synthase expression in interferon-gamma plus LPS-stimulated murine macrophages. We hypothesize that DHA may inhibit NF-κB activation, and consequently TNF-α mRNA expression, by triggering antioxidant systems. In addition, DHA effect could be linked to D-series resolvins, DHA-derived lipid mediators with anti-inflammatory activity[15, 62].

In the absence of IL-23 stimulus, LA promoted an increase of TNF-α mRNA expression with respect to unsupplemented cells, according to other authors[41, 42]. In IL-23 treated cells the pro-

inflammatory effect of LA was not either additive or synergic to IL-23. The full nature of this result needs further investigation.

Overall, our data suggest some food bioactive compounds as possible anti-inflammatory agents, able to break down the newly identified inflammatory loop involving IL-23, IL-17 and all cytokine network. In some cases, tested bioactives were more effective than sodium salicylate in the modulation of pro-inflammatory IL-23-mediated stimulus.

It is interesting to note that all tested bioactives are recognized as peroxisome proliferator-activated receptor (PPAR) ligands, and it is conceivable that their inhibitory effect results from a PPAR-mediated inhibition of NF-κB signaling pathway. Among PPAR isoforms, PPAR-γ is highly expressed in the colon, mainly in the crypts and surface of epithelial cells[63], as well as in macrophages and T and B lymphocytes[63-71]. Recently, ligands for the PPAR-γ have been shown to inhibit the expression of various cytokines in monocytes and macrophages, principally by preventing the activation of NF-κB by an unclear mechanism[72, 73]. However, PPAR-γ levels are decreased during chronic inflammation in humans and animals[65-71, 74, 75].

TNF-α is known to inhibit the ligand-dependent transcriptional activity of PPAR-γ with a mechanism not fully understood[76]. Inhibition of PPAR-γ function by TNF-α can represent a molecular mechanism of the inflammation disorders, such as IBD. Recent reports indicate that PPAR-γ ligands decrease the degree of inflammation associated with experimental colitis[70, 71, 77-79]. In fact, 5-aminosalicylic acid (5-ASA), one of the oldest anti-inflammatory chemicals used for the treatment of IBD, binds PPAR-γ, thus explaining its anti-inflammatory effects in the colon[80]. In this perspective, bioactives PPAR-γ ligands could represent a novel approach to the treatment of IBD[70].

In conclusion, targeting IL-23 receptor and his downstream signaling pathways, including PPAR-γ, could have preventive and therapeutic role against chronic inflammatory disorders, such as IBD.

In this light, some bioactives present in foods could be a new, interesting approach for the prevention and treatment of IBD.

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