



## Synergistic Antioxidant Activity of Natural Products

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### Abstract

The mixture of bioactive constituents and their byproducts contained in plant extracts and various natural products produce synergistic effects. But the mechanisms responsible for synergistic antioxidant activity are not explained yet due to the complex nature of the plant extracts. The mechanisms underlying synergistic interactions should be known for the standardization and optimization of mixtures of different synthetic and natural antioxidants and also for the formulation of different natural antioxidants like single extracts or multi-extract preparations which requires quick and reliable analytical methods, evaluation mechanisms and an easy, clear and unbiased method of synergy assessment. The synergistic antioxidative activity of the optimized mixture can more effectively or at lower doses, prevent or treat the various diseases caused by oxidative damage and also protect food from oxidation and microbial contamination thus reducing the side effects caused by the excessive use of synthetic antioxidants.

### Introduction

Free radicals are produced as a normal part of metabolism. Antioxidants maintain delicate balance between the production and neutralization of ROS, but if this balance is disturbed, the cells start to suffer the consequences of oxidative stress [1]. Which leads to the development of several chronic diseases including cancer, cardiovascular diseases including atherosclerosis and stroke, neurological disorders, renal disorders, liver disorders, hypertension, rheumatoid arthritis, adult respiratory distress syndrome, auto-immune deficiency diseases, inflammation, degenerative disorders associated with aging, diabetes mellitus, diabetic complications, cataracts, obesity, autism, Alzheimer's, Parkinson's and Huntington's diseases, vasculitis, glomerulonephritis, lupus erythematosus, gastric ulcers, hemochromatosis and preeclampsia [2-4]. Free radical mediated damage to lipids, proteins, and DNA is mainly responsible for these negative effects [5]. Although the human body produces antioxidant enzymes to neutralize free radicals, exogenous antioxidants are required to assist the human body to protect it self [6]. Antioxidants can be defined as "any substance that, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate" [7], but later the definition comes out as "any substance that delays, prevents or removes oxidative damage to a target molecule" [8]. In the same year the definition of antioxidants was given as "any substance that directly scavenges ROS or indirectly acts to up-regulate antioxidant defences or inhibit ROS production" [9].

Different antioxidants follow different mechanisms or pathways under various stress conditions in order to show their antioxidative response. The main mechanisms involve: inhibiting free radical oxidation reactions (preventive oxidants), interruption of propagation of the autoxidation chain reaction (chain breaking antioxidants); inhibiting formation of free lipid radicals; quenching single oxygen species; reducing hydro peroxides converting them into stable compounds; inhibiting pro-oxidative enzymes; chelating metals and converting metal-pro-oxidants into stable products and through synergism with other antioxidants [10-14].

In this short review, the focus is on synergism in antioxidant activity. Synergism comes from the Greek word "synergos" meaning working together. Synergism can be defined as the coordinated or correlated action of two or more structures, agents, or physiologic processes so that the combined action is greater than the sum of each acting separately. While the mechanisms of synergism can change from situation to situation, most of the time there appears to be an effect on the enzymes. With synergism, an enzyme function could either be inhibited (restricted) or accelerated in some way. Either way, the result is that the chemicals are either "free" or "enhanced" to cause a greater effect. Generally the drug combinations have proven to be an essential feature of antimicrobial and antioxidant treatment due to a number of important considerations viz. (i) they increase activity through the use of compounds with synergistic or additive activity; (ii) they thwart drug resistance due to the presence of multiple active components having diverse structures and functional groups

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(iii) they decrease required doses, reducing both cost and adverse/toxic side effects because different components in the mixture reinforce or complement the activity of each other and (iv) they reflect broad spectrum of activity. Natural products like plant extracts and essential oils are the good source of natural antioxidants and antimicrobial compounds. They are preferred over the synthetic antioxidants (BHT and BHA) due to their multiple beneficial effects which are produced by the presence of a wide spectrum of antioxidant components in them. Moreover, synergistic or increased effect in the antioxidant and antimicrobial activities has been shown by the mixture of different natural products like plant extracts and essential oils and also by the combination of natural and synthetic antioxidants [15]. Since the oxidation process follows a complicated set of mechanism, mixture of compounds (phytochemicals or synthetic) would have greater bioactivity than a single compound because a mixture of bioactive compounds has the ability to affect multiple targets [16] and also the activity of one antioxidant is dependent on the other.

Antioxidants have a wide range of biological and pharmacological activities and are considered to be of great benefit in nutrition and health [17]. These can also prevent or delay the oxidation of food and thus are used in food preservation [18] because these natural antioxidants avoid undesired health problems that may arise from the use of synthetic antioxidants. Spices and herbs acting as natural antioxidant and antimicrobial agents, can prevent food spoilage caused by oxidation and microbial action. But these are effective only when present at high concentrations which may lead to alteration in the taste, colour, odour and texture of foods and thus limit their use in food preservation system [19]. This limitation can be eliminated by using a combination of herbs. This approach may take advantage of possible synergistic interactions in which antimicrobial and antioxidant efficacy of the combination of herbs increases at sufficiently low concentration. This synergistic interaction may reduce their adverse side effects which are caused by the higher concentrations of a single herb. It was studied that some polyphenolic components of green tea exhibited strong synergistic antioxidative activity with tocopherol [20]. Total antioxidant capacity in vegetables, fruits and their processed products is the net result of synergistic effect, negative and additive effect [21-24]. Several hypotheses have been proposed to explain the mechanisms of synergism and antagonism. Some antioxidants in combination act in a regenerating manner, with either the stronger regenerating the weaker, antagonistic effect or the weaker regenerating the stronger, synergistic effect [25]. Other features are also involved in the interaction of compounds in combination, which include reaction rates of antioxidants, the polarity of the interacting molecules and the effective concentration of the antioxidants at the site of oxidation [26-28]. The interactions among different antioxidant components can be synergistic (the combined effect of two components is much greater than the sum of the effects of each agent given alone), additive (the combined effect of two or more components is equal to the sum of the effect of each agent given alone) or antagonistic (opposite of synergism). It may be successful to use antioxidant combinations in which the antioxidants produce a synergistic effect.

## Assessment of Synergy

Only a few well-documented instances of synergy assessment are available in the literature, and there are several reasons for this, the main one being the difficulty in methodology of proving such effects. It requires testing the activity of individual constituents and comparing the activity with an equivalent dose in the mixture. This

improved and co-operative sort of effect can be called as "polyvalent or pleiotropic action". It is not easy to define and prove synergy [29-34]. The general understanding of synergy is that it is an effect seen by a combination of substances being greater than would have been expected from a consideration of individual contributions. Synergy can be described by either an increased therapeutic effect, a reduced profile of side effects or, preferably (and logically), both. But it is very difficult to prove or describe synergy accurately in plant extracts or herbal mixtures due to very less knowledge about the chemical nature or quantity of the various constituents. Berenbaum (1989) gave the "isobole" method to describe and prove synergy effects. Isobole method is the most adapted method for proving synergy interactions. This method is independent of the mechanism of action. It can be applied to multiple component mixtures like herbal mixtures or plant extracts as it makes no assumptions to the behavior of each agent. An isobole is a graphic demonstration in which the dose rates of the single individual components are represented on the linearly arranged x and y axes. The dose combinations are represented by geometric points with coordinates matching the dose rate of the separate components in the combination. A line or curve between points of the same effect is known as isobole or is effect curve. If synergy is occurring, i.e. the effect of the combination is greater than expected from their individual dose-response curves, the dose of the combination needed to produce the same effect will be less than for the sum of the individual components and the curve is said to be 'concave'. The opposite applies for antagonism, in which the dose of the combination is greater than expected, and produces a 'convex' isobole. It is quite possible that one dose combination produce synergistic interactions while the another produce antagonism with the same substances giving rise to a complicated isobole with a wave-like or even elliptical appearance.

## Synergistic Studies of Antioxidant Activity

Synergism in antioxidant activity can be discussed as: Synergistic interactions between the antioxidant components from various natural products; synergism between antioxidant components in different solvent fractions of same plant extract or mixture of different herbal preparations; and synergism between synthetic antioxidants and natural products.

## Synergistic Interactions Between the Antioxidant Components from Various Natural Products

In binary mixture, the interaction between phenolic antioxidants produce synergistic effects with the combination of rosmarinic acid and quercetin, or rosmarinic acid and caffeic acid, whereas antagonistic effects were obtained with the  $\alpha$ -tocopherol/caffeic acid;  $\alpha$ -tocopherol/rosmarinic acid; (+)-catechin/caffeic acid; and caffeic acid/quercetin in the 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced oxidation [35]. The term hurdle technology has been applied to the use of plant extracts for inhibiting the oxidation and growth of food borne pathogens. This is an acceptable method of food preservation in industries [36]. Plant extracts have multiple antioxidant and antimicrobial constituents which provide a broad range of antioxidant and antimicrobial activity. This enhanced or broad activity may be produced due to the interaction of two or more compounds in the combination [37-38]. The synergistic antioxidant activity was also observed in model oil-in-water emulsions containing combination of bovine serum albumin (BSA) and each of the green tea catechins [epicatechingallate (ECG), EGCG, EC and epigallocatechin

(EGC)]. Although BSA had very little antioxidant activity in the absence of phenolic antioxidants, the combination of BSA with each of the catechins showed strong antioxidant activity after 45 days storage [39]. Synergistic antioxidant activity is dependent on the type of antioxidant and its concentration. Lycopene interacted synergistically with vitamin E at a specific concentration and ratio to inhibit the AMVN (2,2-azobis (2,4-dimethylvaleronitrile) induced oxidation of LAME (Linoleic acid methyl ester) whereas  $\beta$ -carotene showed no synergistic effect with vitamin E at the same levels [40]. In biological systems, synergy of antioxidants is influenced by various factors. The appropriate concentration and combination of antioxidants appear to be important factors for maximum antioxidative synergistic action. Papaya and strawberry nectars and all their respective blends (25P:75S, 50P:50S, 75P:25S) showed synergistic relationship for ascorbic acid and  $\beta$ -cryptoxanthin after processing by ultra high temperature (UHT) and irradiation respectively. Both antagonistic and additive relationships were seen for  $\beta$ -carotene whereas lycopene concentration encountered synergistic relationships in only 25P:75S blend for both techniques. All blends exhibited synergistic relationships for antioxidant capacity after UHT processing. These findings demonstrate the benefits of blending fruit nectars; producing a superior product [41]. Antioxidant level in chickpea seeds was improved by the synergistic interaction of microbes in the rhizosphere. The triple and double combination of microbes fluorescent *Pseudomonas* (PHU 094), *Trichoderma harzianum* (THU 0816) and *Mesorhizobium* sp. (RL 091) produce significantly higher total phenolic and flavonoid content, ascorbic acid, free radical and hydroxyl radical scavenging activities as well as reducing power in seeds and pericarp compared to their single application [42]. The synergistic antioxidant activity was also shown by the extracts of mushrooms *Suillus luteus* (Sl) and *Coprinopsis atramentaria* (Ca) on combining them in the ratios of Sl:Ca (1:1) and Sl:Ca (2:1). A higher synergistic effect or lower EC50 value was obtained for the combinations in DPPH radical scavenging, reducing power, and  $\beta$ -carotene bleaching inhibition assays [43]. It is impossible to explore the potential interactions with in the phenolic compounds in order to better understand the total antioxidant capacity of samples as phenolic compounds are usually present in the form of mixtures. Information about the interactions and potential synergistic or antagonistic effects between different phenolic compounds is lacking. In addition, one method cannot fully describe the antioxidant activity of the samples, and multiple method approach is necessary for the full of the antioxidant properties of the samples [44-45]. Three different antioxidant assays (FRAP, DPPH, and Briggs–Rauscher reaction) were used to study the interaction between resveratrol and other phenolics (gallic acid, caffeic acid, catechin, quercetin) in equimolar binary mixtures. The obtained results indicate that there are differences in the activity of mixture of phenolic compounds and resveratrol depending on mechanism of the used method. All three methods confirmed the synergistic effect between catechin and resveratrol. The reducing activity of the mixture of caffeic acid and resveratrol was almost 10.0% higher than the activity of the individual compounds, while antagonistic effect was observed in this phenolic mixture in the other two assays that evaluate free radical scavenging activity of the samples. Briggs- Rauscher reaction detects that inhibition time is significantly prolonged and the effect was greatly enhanced in the mixtures of gallic acid and resveratrol and of catechin and resveratrol, by 45.5 and 53.3% respectively. The obtained results support the fact that the antioxidant activity of phenolic mixtures cannot be predicted from the activity of pure compounds, as the bioactive compounds

do not act indecently but they could be useful in the explanation of the activity of their mixtures and helps in making a conclusion about their combined effects, synergistic or antagonistic, on the antioxidant properties of the samples [46]. The obtained results confirmed that the activities of the tested compounds vary depending on their structure, concentration, time and the used method, but generally, like in previous studies, similar rank of activities was obtained [47-51].

## Synergism between Antioxidant Components of Different Herbs

It has been reported that a combination of herbs show a synergistic antioxidant activity. The synergistic antioxidant activity of green tea with some herbs to support the hypothesis that formulation of a polyherbal combination of plants having antioxidant activity (*Vitis vinifera*, *Phyllanthus emblica* L., *Punica granatum*, *Cinnamomum cassia*, *Ginkgo biloba* L., and *Camellia sinensis* Linn.) shows a synergistic effect with green tea. It was found that the antioxidant activity synergistically increases by the action of all these herbs in combination with green tea and thus lower doses of each herb with green tea may be used. Study reported that selected individual plants contained abundant quantity of phenolics and flavanoids and their polyherbal combination with green tea was found to produce best antioxidant activity among all individual extracts. This will help in avoiding undesirable side effects due to higher doses of single herb [52]. Two effective fractions (C-3 and D-2) from the acetone crude extracts (A) of *Potentilla fruticosa* leaves were obtained by bio-guided chromatographic fractionation. Isobolographic analysis indicated that the combination of A with EGb (acetone extract of *Ginkgo biloba*) showed best synergistic activity at the ratio of 5:1 (A+ EGb). C-3+ EGb showed synergism in DPPH and ABTS assays whereas additive effect in FRAP assay. While only additive effect was shown by D-2 + EGb [53]. Antibacterial and antioxidant efficacy of essential oils of some selected spices and herbs [bay leaf, black pepper, coriander (seed and leaf), cumin, garlic, ginger, mustard, onion and turmeric] in combination showed synergistic interactions. The evaluation of different possible combinations of these herbs showed that only coriander/cumin seed oil combination produce synergistic interactions both in antibacterial (FICI : 0.25-0.50) and antioxidant (CI : 0.79) activities whereas other combinations showed additive effects (CI = 1.00) which indicates that the proton donating ability of coriander /cumin seed oil combination is high at low concentration over other tested combinations. The bioactive compound was isolated and identified by TLC –bioautography guided screening. From the Rf values and chemical analysis, the bioactive compound present in coriander oil was found to be linalool (Rf = 0.35) and the compound present in cumin seed oil was identified as p-coumaric acid (0.53). It was found from the combination studies that these two compounds are responsible for both synergistic antibacterial and antioxidant activities [54]. Antioxidant assays like 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) free radical scavenging assay and ferric reducing anti-oxidant power (FRAP) assay were used to assess the antioxidant activity of different solvent extracts of *Astragalus membranaceus* (AME), *Glycyrrhiza uralensis* (GU) and the combination of them (AG). A higher antioxidant activity was shown by the ethyl acetate fraction of the combination of extracts than the sum of two single herbs and the combination also showed better cytotoxicity activity. There is difference in the antioxidant activity of different solvent extracts of AME, GU and AG in ferric reducing

anti-oxidant power. Highest FRAP was observed in the chloroform extracts of AME (CF-AME) and GU(CF-GU). However, the combination of both extracts (AG) showed highest FRAP in the ethyl acetate fraction (EA-AG) i.e in more polar fraction than chloroform. Ethyl acetate fraction of the combination of herbs (EA-AG) showed the highest antioxidant activity in DPPH and ABTS assays also [55]. Synergistic antioxidant effect was shown by infusions prepared with the plants (either *Pterospartumtridentatum* and *Cymbopogoncitratu*; or *G. globosa* and *Cymbopogoncitratu*) in proportion 40%: 60% [56]. The combined mixture of honey and mint (*Menthaspicata*) produced synergistic antioxidant activity in DPPH, ABTS and reducing power assays [57].

## Synergism between Synthetic Antioxidants and Natural Products

The oxidation of methyl linoleate can be inhibited by the synergistic interaction of nucleic acids (DNA and RNA) and tocopherols. Nucleic acids form hydrogen bonds with tocopherols and protect them from the direct oxidation in the presence of oxidizing methyl linoleate [58]. A synergistic antioxidant effect between RE (methanol rosemary extract) and BHT (butylated hydroxytoluene) was demonstrated by isobolographic analysis. A synergistic antioxidant effect between RE and BHT was demonstrated by isobolographic analysis and a synergistic interaction of RE with BHA to inhibit *Escherichia coli* and *Staphylococcus aureus* growth was observed. Methanolic rosemary extract (RE) not only enhances the antioxidant efficiency of BHA and BHT, but also the antibacterial effect of BHA; allowing a decrease from 4.4 to 17 folds in the amounts of the synthetic compounds used. Therefore rosemary extract can be used for preventing both food oxidation and the growth of food borne pathogens thus reducing the side effects caused by the high concentrations of synthetic antioxidants [59]. The combinations involving BHA and four phenolic compounds (hydroquinone, carvacrol, thymol, gallic acid and octylgallate) studied were mostly additive, with the exception of BHA + carvacrol and BHA + thymol that were antagonistic [60]. Potential synergistic activity was found in combinations of natural antioxidant (NAO) isolated from spinach leaves and commercial antioxidants. Synergistic antioxidant activity was found with three polyphenols – ferulic acid, caffeic acid, and epigallocatechin-3-gallate [61]. These findings demonstrate the importance of using antioxidant ‘cocktails’.

## Conclusion

Natural products like plant extracts and essential oils are widely used all over the world due to their antioxidant activity which protect human beings from various diseases caused by oxidative stresses. But their true potential has not been explored yet. Antioxidant potential can be increased by the synergistic interactions between different antioxidant compounds present in the mixture of synthetic antioxidants and natural products or the mixture of different herbal extracts or plant essential oils. The synergistic interactions decrease the requirement of doses of different drugs in combination thus reducing the side effects caused by the high concentrations of a single drug. But very little is known about the mechanism of interactions which are responsible for the synergistic antioxidant activity. Because a greater understanding of the exact mechanisms of action of each and individual component of the plant extracts/essential oils is required to describe and prove synergy. Moreover only one analytical method cannot fully describe the antioxidant activity of the samples, and multiple method approach is necessary for the full evaluation of the

antioxidant activity. Due to the complexity of plant extracts, a huge analytical effort and manufacturing skills are required to produce well-defined, standardized herbal preparations. Synergistic interactions are very sensitive and depend on the types of different compounds in the combination and also on their relative proportions. A particular combination may show synergistic behavior at one concentration ratio while antagonistic behaviour at the other. So the types of different compounds and their respective concentrations in the mixture requires a proper standardization so that the maximum synergistic activity of the mixture can be achieved. Most of the combinations have not been tried yet, only a very small percentage of the combinations is tried. So a large number of synergistic combinations are still unexplored. There is also a need for the development of new methods for pharmacological studies and clinical trials evaluating the effects produced by complex mixtures of compounds. Much information about the mechanisms of synergistic effect can be gained by in vivo studies but these approaches are not commonly used. Most of the evaluation mechanisms are still depend on the in vitro approaches. Use of different synergistic combinations can also help to decrease the dependence on synthetic antioxidants and other chemicals that cause adverse effects on the living beings and environment due to their very slow breakdown responsible for biomagnifications. A lot of research work is still required to explore the full synergistic potential of numerous mixtures obtained by various combinations which has not been tried yet. This requires optimization of the combinations by quick and reliable analytical methods, evaluation mechanisms and an easy, clear and unbiased methods of proving and assessing synergy. The optimized combinations are highly efficient and can be regarded as super antioxidants or new generation phytoantioxidants.

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